Regulation of Spermatogonial Stem Cell Maintenance and Self-Renewal

5

Tessa Lord and Jon M. Oatley

Abstract

Spermatogonial stem cells (SSCs) reside within the stem cell niche along the basement membrane of the seminiferous tubules in the testis, and their actions provide the basis for continuity and regeneration of the spermatogenic lineage. SSCs must balance self-renewal with the production of progenitor spermatogonia in order to sustain optimal sperm production while preventing exhaustion of the stem cell reservoir. Regulation of SSC fate decision is in part influenced by signaling from growth factors, such as Gdnf and Fgf2, which are synthesized by somatic niche support cells. Such growth factors have been shown to directly influence expression of transcription factors such as Id4, Etv5, and Bcl6b within SSCs to stimulate self-renewal. Additionally, the undifferentiated state of both SSCs and progenitors is maintained by virtue of intracellular regulation at transcriptional, translational, and posttranslational levels; both independently and dependently of characterized growth factors released from the niche. This intrinsic regulation not only acts to enrich the expression of genes important for maintaining the undifferentiated state, but also supresses expression of differentiation-driving factors. Although progress in SSC research has previously been dampened by a lack of SSC-specific markers that can be used to isolate pure populations for analysis, recent advances have seen the development of mouse lines in which the SSC population alone is marked by expression of a fluorescent reporter transgene; for example the *Id4-eGfp* mouse line. Consequently, in-depth analysis of the SSC population in comparison to undifferentiated progenitors and differentiating spermatogonia is now possible. Further progress in characterizing factors involved in SSC maintenance and self-renewal is important for

T. Lord • J.M. Oatley (✉)
Centre for Reproductive Biology, School of Molecular Biosciences, Washington State University, Biotechnology/Life Sciences Building, 1770 NE Stadium Way, Pullman, WA 99164, USA
e-mail: joatley@vetmed.wsu.edu

J.M. Oatley, M.D. Griswold (eds.), *The Biology of Mammalian Spermatogonia*,
https://doi.org/10.1007/978-1-4939-7505-1_5

understanding potential underlying causes of idiopathic infertility, and further, is the basis for developing therapeutic strategies aimed at reinstating fertility in patients who have been rendered infertile as a consequence of chemotherapeutic treatments in pre-pubertal life.

Keywords
Spermatogonial stem cells • SSC niche • SSC self-renewal

5.1 Introduction

The foundation for continual and robust spermatogenesis in the mammalian testis is provided by actions of spermatogonial stem cells (SSCs). Mitotic division of SSCs is the basis for self-renewal that underlies maintenance of a foundational pool. From this pool, progenitor spermatogonia arise that amplify in number, also through mitotic divisions, prior to transitioning to a differentiating state under the influence of retinoic acid; an event that signifies commitment to terminal differentiation as spermatozoa (detailed in Chap. 6). In adulthood, SSC activities fuel the production of approximately 70 million sperm per day in rodents, and 100 million sperm per day in humans (Johnson et al. 1980). Historically, the rarity of SSCs in the testis [approximately 0.03% of cells in mouse and 1.25% of cells in human testes (Tagelenbosch and de Rooij 1993; Aponte et al. 2005)], in conjunction with a lack of established molecular markers available to distinguish SSCs from progenitor cells, has made the elucidation of pathways responsible for stem cell maintenance and self-renewal difficult. Fortunately, recent advances in the field, particularly the identification of factors expressed exclusively in SSCs, have paved the way for critical advances in our knowledge of these processes.

5.2 Developmental Origins of the SSC Population

SSCs originate from prospermatogonia that differentiate from primordial germ cells (PGCs) on the genital ridge during embryonic development. The majority of our understanding of this process comes from mouse studies which will be summarized here, while events in humans will be touched on briefly. Specification of PGCs is thought to begin from embryonic day 6–6.5 (Yoshimizu et al. 2001; Saitou et al. 2002), and complete specification can be identified by day 7–7.5; at which time expression of germ cell-specific markers, such as alkaline phosphatase, can be visualized (Chiquoine 1954; Ginsburg et al. 1990). In conjunction with the specification process, PGCs experience epigenetic reprogramming, including global gene demethylation and histone modification (Tseng et al. 2015). Following these events, PGCs undergo extensive mitotic proliferation up until embryonic day 13.5 (Nakatsuji and Chuma 2001). At this time, differentiation according to embryonic sex occurs, with PGCs in XY embryos transforming into prospermatogonia (De Felici et al. 2004).

The prospermatogonia continue to proliferate until day 15.5 at which point a period of quiescence is initiated and sustained until neonatal development (1–5 days postpartum depending on strain of mice) (Kluin and de Rooij 1981; Ginsburg et al. 1990). During the quiescent period, a new DNA methylation profile is initiated within prospermatogonia (Tseng et al. 2015), becoming fully established by the time of reentry into the cell cycle. Concomitant with cessation of de novo DNA methylation and resumption of proliferation, prospermatogonia transform into a primary population of undifferentiated spermatogonia comprised of SSCs and progenitors, and a differentiating population of spermatogonia that give rise to the first round of spermatogenesis at postnatal days 28–35. All other rounds of spermatogenesis arise from the primary undifferentiated population (Yang et al. 2013b).

As humans have a considerably longer pre-pubertal period than their murine counterparts, the transition of prospermatogonia into a population of postnatal spermatogonia does not occur until 2–3 months of age, while differentiation is not observed until approximately 12 years of age (Paniagua and Nistal 1984). The intricate transformation of prospermatogonia into spermatogonia during embryonic and neonatal life is clearly imperative for the formation of an SSC population that fuels sperm production in adulthood; however, impairment of this transition is also associated with pre-neoplastic changes that are thought to be the root of carcinoma in situ cells: the major source of testicular germ cell tumors (Skakkebæk 1972). Our understanding of the mechanisms and potential models for specification of the SSC pool from prospermatogonial precursors is detailed further in Chap. 2.

5.3 Models for Maintenance and Renewal of the SSC Pool

Following the onset of puberty, continuity of the spermatogenic lineage requires maintenance of the SSC pool. During steady-state conditions, progenitor spermatogonia periodically arise from SSCs; however, this is balanced by self-renewal to prevent depletion of the SSC population. Alternatively, certain situations require rapid regeneration of the spermatogonial population; such as following a cytotoxic insult that depletes a major portion of the population, in response to spermatogonial transplantation, or during neonatal development. In these circumstances self-renewal must predominate to rebuild the SSC pool. Again, in the absence of self-renewal, the SSC population will decline over time and eventually become exhausted, or regeneration will be stunted.

The most widely accepted model depicting the dynamics of the SSC and progenitor spermatogonial pools is referred to as the "A_{single} model". In rodent species, spermatogonia exist in three subclasses; type A, intermediate, and type B. It is well established that SSCs are a component of the type A spermatogonia, in particular the undifferentiated subpopulation. Mitotic division of the SSCs may either be symmetrical; producing two new SSCs (or two progenitors), or alternatively, asymmetrical; producing one SSC and one progenitor cell that will be committed to differentiation (Fig. 5.1a). Upon mitotic proliferation, progenitor cells remain connected by persistent cytoplasmic bridges, forming structures referred to as "A_{paired}"

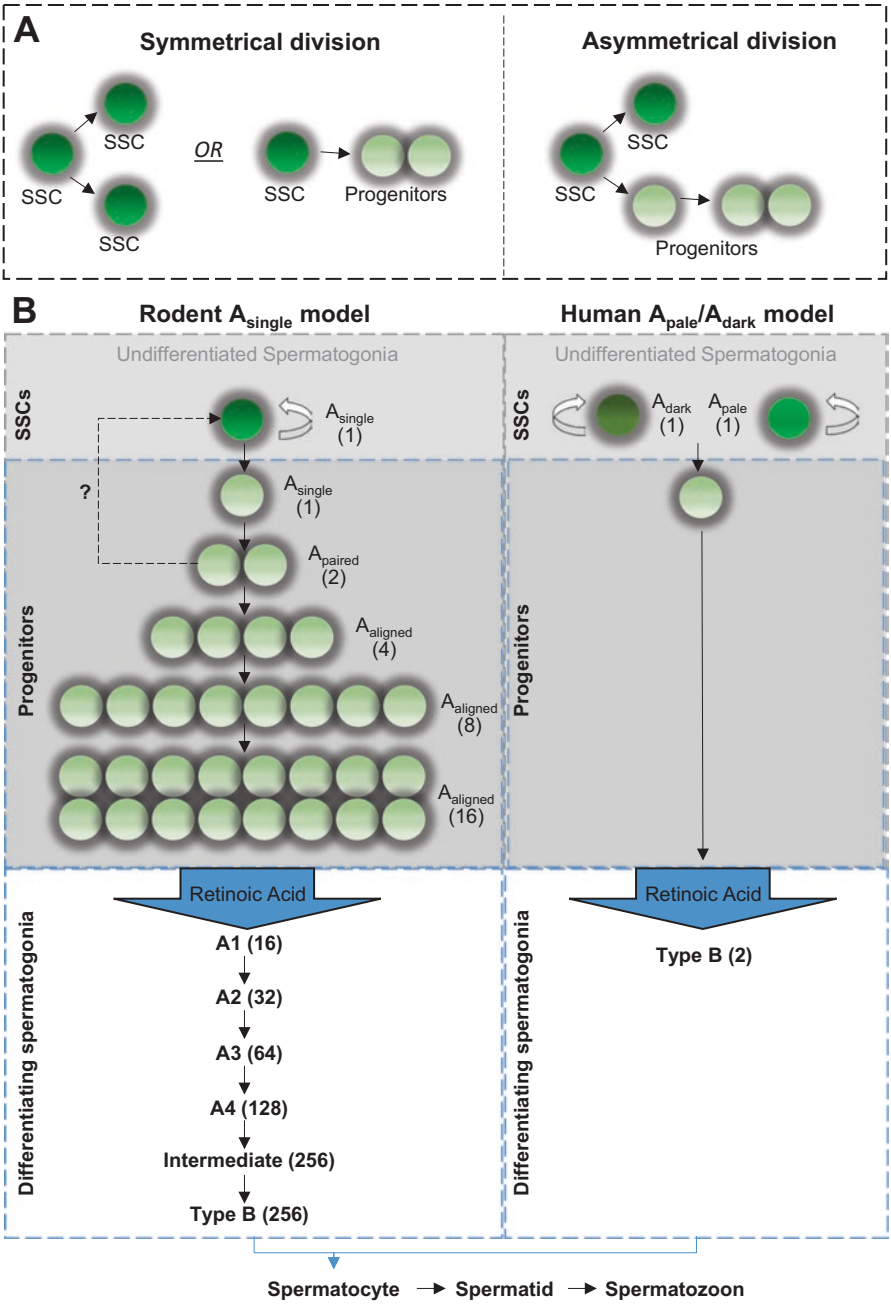

Fig. 5.1 Maintenance and renewal of the SSC pool. (**a**) Depending on systemic requirements, SSCs may either favor self-renewal to replenish the population, favor progenitor formation for the production of sperm, or maintain steady-state conditions. Thus, mitotic division of SSCs may be either symmetrical or asymmetrical. (**b**) The most widely accepted model for SSC maintenance and

spermatogonia. As a consequence of this syncytial connection, further mitotic divisions of these progenitors occur in synchrony, producing chains of spermatogonia referred to as "$A_{aligned}$," consisting of up to 16 cells (Fig. 5.1b). Cumulative evidence derived from rodent studies suggests that stem cell activity resides exclusively within the isolated A_{single} population, with the formation of an A_{paired} structure signifying commitment to a differentiation pathway. The first experimental evidence supporting this model came in the form of morphological and proliferative observations on spermatogonia in rodent testes in the 1970s (Huckins 1971; Oakberg 1971) and has been supported by more recent research efforts that utilize fluorescent reporters to identify SSCs specifically amongst the heterogeneous population of spermatogonia in the testes (Chan et al. 2014). Although the 'A_{single}' hypothesis depicts that all SSCs exist as A_{single} cells, conversely, not all A_{single} cells are believed to be SSCs. In fact, it has been estimated that under 10% of the A_{single} population retains the capacity for self-renewal and regeneration of the spermatogenic lineage (Nagano 2003; Chan et al. 2014). Thus, a 'revised' A_{single} model has been put forth to factor in this nuance (reviewed by Lord and Oatley 2017).

In contrast to the traditional A_{single} model, a recently emerged hypothesis, the 'fragmentation' hypothesis, proposes that contribution to the stem cell pool may be a more dynamic process than originally believed (reviewed by Lord and Oatley 2017). Time-lapse imaging studies have been used to demonstrate that spermatogonia can break off from $A_{aligned}$ chains to produce single cells (Hara et al. 2014); however, whether these cells, which can now be identified as "A_{single}," possess the functional attributes of an SSC remains to be unequivocally demonstrated. Despite this, results of pulse-chase experiments have suggested that a small subset of progenitors may be able to revert to a stem cell state and induce colonization in the testes. This occurrence is purportedly more common when regeneration of the germline is required (in this case, following transplantation or tamoxifen exposure), rather than during steady-state conditions (Nakagawa et al. 2010). Potentially, these data may depict that a subset of the rodent undifferentiated spermatogonia population act as "reserve" or "potential" stem cells, that contribute to the self-renewing population when replenishment of the germline is required; however, do not significantly contribute to the self-renewing population in steady-state conditions.

Fig. 5.1 (continued) progenitor production in rodents is the A_{single} model in which the A_{single} spermatogonia make up the SSC population. Upon transition into a progenitor state, cytokinesis following mitotic division is incomplete, forming pairs (A_{paired}), and chains ($A_{aligned}$) of spermatogonia. Undifferentiated progenitors commit to differentiation in response to retinoic acid signaling. Differentiating "A1" spermatogonia transition further to A2, A3, A4, Intermediate, and Type B spermatogonia, accompanied by additional mitotic divisions, before finally committing to the initiation of meiosis at the spermatocyte stage. Following two rounds of meiosis, haploid spermatids are formed that will mature into spermatozoa. In contrast to the rodent model, the human model for SSC maintenance is the A_{pale}/A_{dark} model. In this system, the A_{dark} cells are thought to be the "reserve" stem cells, while the A_{pale} cells actively contribute to fueling steady-state spermatogenesis. The primary difference between the rodent and human undifferentiated spermatogonial pool is the absence of rounds of successive mitotic division of progenitors in the human model. Instead, progenitor spermatogonia produced from division of A_{pale} or A_{dark} SSCs transition directly into Type B differentiating spermatogonia

In contrast to rodent species, the paradigm depicting SSC dynamics in primates is referred to as the A_{pale}/A_{dark} model (Fig. 5.1b). The primate type A spermatogonial population has been divided into these two subsets as a reflection of the different staining intensity of nuclei of spermatogonia by hematoxylin (Clermont and Antar 1973). Although not definitively classified with functional evidence, both A_{pale} and A_{dark} cells are thought to possess stem cell activity, with A_{dark} cells considered to be the reserve or "back-up" stem cells, while the A_{pale} cells actively self-renew and contribute to spermatogenesis in steady-state conditions (Clermont 1969). Progenitor cells produced from the A_{pale} division transition directly into type B spermatogonia (Clermont 1966), thus eliminating the clonal expansion step that is observed within mouse spermatogenesis. The smaller number of mitotic divisions during spermatogenesis in primates means that this process is highly inefficient when compared to that of rodents; particularly in the case of humans, whose spermatogonial progenitor cells undergo only one mitotic division prior to differentiation into a type B spermatogonium (Amann 2008).

5.4 The SSC Niche

Maintenance of the SSC pool is reliant on a niche microenvironment within the testis that is comprised of contributions from somatic cells that provide structural support as well as secrete growth factors that regulate the balance between self-renewal and differentiation. As mentioned previously, the requirement for intensive SSC renewal during neonatal development is a distinct contrast to the steady-state conditions required for maintenance during adulthood; representing the intricate and dynamic nature of the SSC niche. The SSC niche is comprised of the germ cells themselves, as well as the Sertoli cells; residing inside the epithelium of the seminiferous tubule (Fig. 5.2).

Observational studies have resulted in postulation that the formation of stem cell niche regions within the testis preferentially occurs adjacent to the blood vessels and interstitium, particularly associating with blood vessel branch points (Yoshida et al. 2007). Despite this, direct functional studies are yet to be conducted to confirm this observation. Also, another study observed that a majority of undifferentiated spermatogonia align in regions of seminiferous tubules associated with the interstitium at stages VII-VIII of the seminiferous cycle (Chiarini-Garcia et al. 2003). Considering that these stages are when most of the undifferentiated spermatogonia transition to a differentiating state, the association with vasculature and interstitial tissue may be driving the differentiating transition rather than influencing maintenance of the SSC pool. Furthermore, Inhibitor of DNA binding 4- (Id4)-eGfp+ spermatogonia which possess potent SSC capacity reside in areas of tubules that are not associated with the interstitial space (Chan et al. 2014). Moreover, experimental alteration of SSC niche number within seminiferous tubules does not alter the amount of area that associates with the vasculature or interstitium (Oatley et al. 2011a). Taking all of these observations into account, further investigation into whether the vasculature and interstitial tissue are key components of the SSC niche is clearly warranted.

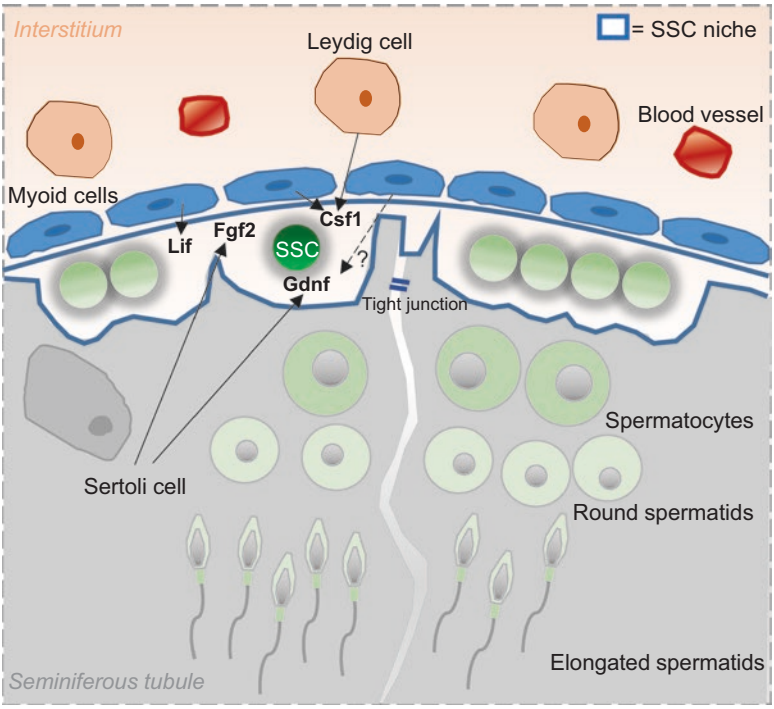

Fig. 5.2 The spermatogonial stem cell niche. The SSC niche-unit is comprised of the SSCs themselves, undifferentiated progenitor spermatogonia, and surrounding somatic support cells. The somatic cells of the SSC niche; the Sertoli, Leydig, and peritubular myoid cells; release growth factors that are thought to stimulate self-renewal of SSCs, and maintenance of an undifferentiated state in both SSCs and progenitors. The Sertoli cells, in addition to providing architectural support to the niche, produce the growth factors Gdnf and Fgf2, while both Leydig and myoid cells produce Csf1, and myoid cells produce Lif, and potentially Gdnf

Although the Sertoli cells are regarded to be the most influential regulators of the stem cell niche due to their direct interaction with germ cells, somatic cells within the interstitial space such as the Leydig and peritubular myoid cells also appear to be involved in niche regulation. Certainly, the positioning of the SSC population on the outer of the blood-testis barrier (formed via tight junctions between the Sertoli cells) would allow for exposure of these stem cells to regulatory factors that may be produced locally in the interstitium. The critical importance of the somatic support cells within the SSC niche is demonstrated by the direct association between their functionality/abundance and fluctuations in the SSC pool. Indeed, increasing the Sertoli cell population in the testes of mice using transplantation techniques has been shown to result in a threefold elevation in SSC number, as well as an improvement in stem cell niche formation following SSC transplantation (Oatley et al. 2011a). Additionally, the deterioration of niche quality, rather than the quality of the SSCs themselves, is thought to be causative of the decline in fertility that is associated with reproductive ageing. As such, SSCs transplanted from sub-fertile aged animals can support proficient spermatogenesis when transplanted into the testis of young animals (Ryu et al. 2006).

Support cell function in the SSC niche is likely to be a direct implication of the endocrine capacity of these cells. Both Leydig cells and Sertoli cells express gonadotropin receptors: these are luteinizing hormone (LH) and follicle stimulating hormone (FSH) receptors respectively. Certainly, repression of gonadotropin release from the anterior pituitary is directly linked with impaired spermatogonial proliferation in neonates (Kanatsu-Shinohara et al. 2004b). In response to gonadotropins, support cells (particularly the Sertoli cells) of both the murine and primate SSC niche have been demonstrated to release growth factors such as glial cell derived neurotrophic factor (Gdnf), that are directly implicated in maintenance of the spermatogonial population (Van Alphen et al. 1988; Crépieux et al. 2001; Kanatsu-Shinohara et al. 2004b; Mäkelä et al. 2014). In addition, circulating gonadotropins stimulate the production of testosterone by the Leydig cells, with testosterone also being hypothesized to promote growth factor production by both the peritubular myoid and Sertoli cells (Gonzalez-Herrera et al. 2006; Chen et al. 2014, 2016), as well as expression of miRNAs by Sertoli cells (Panneerdoss et al. 2012), that are potentially involved in SSC maintenance (Niu et al. 2011; He et al. 2013).

Perhaps the most extensively characterized paracrine regulator of SSC renewal is the Sertoli-secreted growth factor Gdnf. Interaction of Gdnf with its receptor complex (Ret receptor tyrosine kinase and Gdnf family α1 [Gfrα1]) that is known to be expressed on the surface of spermatogonia (He et al. 2007), not only supports maintenance of the SSC population, but concurrently inhibits differentiation of undifferentiated spermatogonia by retinoic acid; as demonstrated by Gdnf overexpression mouse models (Meng et al. 2000). Further, although *Gdnf*-, *Gfrα1*- and *Ret*-null mice do not survive beyond the first day of postnatal life (Schuchardt et al. 1994; Moore et al. 1996; Pichel et al. 1996; Enomoto et al. 1998), the deleterious effects of decreased Gdnf expression by Sertoli cells have been observed in $Gdnf^{+/-}$ mice. While these mice remain fertile, a high percentage of their seminiferous tubules contain impaired spermatogenesis due to depleted/reduced proliferation of spermatogonia (Meng et al. 2000). Like Gdnf, fibroblast growth factor 2 (Ffg2) is known to be secreted by the Sertoli cells in vivo (Chen and Liu 2015), and is thought to act along with Gdnf in a ratio-dependent manner to regulate SSC maintenance and self-renewal (Takashima et al. 2015). In conjunction with these growth factors secreted by the Sertoli cells; both Leydig and peritubular myoid cells in the mouse exhibit in vivo expression of colony stimulating factor-1 (Csf1), a ligand known to bind to the Csf1 receptor (Csf1r) expressed by a subset of undifferentiated spermatogonial population to enhance SSC renewal in vitro (Oatley et al. 2009). Additionally, peritubular myoid cells produce the growth factor leukemia inhibitory factor (Lif) (Piquet-Pellorce et al. 2000) which may work alongside Gdnf to stimulate SSC proliferation (Wang et al. 2014). Further, peritubular myoid cells may also potentially produce Gdnf themselves (Chen et al. 2016). Indeed, in mice with a Gdnf conditional knockout in peritubular myoid cells, an age-associated reduction in fertility is observed, purportedly as a consequence of the population of undifferentiated spermatogonia being reduced (Chen et al. 2016). A schematic representation of growth factor regulation within the stem cell niche by Sertoli, Leydig and peritubular myoid cells is provided in Fig. 5.2, and an extensive list of growth factors and their purported effects listed in Table 5.1.

Table 5.1 Extrinsic regulatory factors involved in directing fate decisions in SSCs and undifferentiated spermatogonia

Regulatory factor	Origin	Biological effect	Species	Reference
FSH and LH	Pituitary	Indirect effects on SSC self-renewal? Potential regulation of Gdnf secretion by Sertoli cells	Mouse Monkey	Mäkelä et al. (2014) and Crépieux et al. (2001) Kanatsu-Shinohara et al. (2004a) and Van Alphen et al. (1988)
Testosterone	Leydig cells	Indirect effects on SSC self-renewal. Stimulates Gdnf production by peritubular myoid cells and Fgf2 secretion by Sertoli cells	Mouse	Chen et al. (2014) and Gonzalez-Herrera et al. (2006)
Gdnf	Sertoli cells, peritubular myoid cells	Drives SSC self-renewal, impairs retinoic acid-driven differentiation	Mouse Rat Rabbit Hamster	Kubota et al. (2004b), Meng et al. (2000), Chen et al. (2014), and Chen and Liu (2015) Ryu et al. (2005) Kubota et al. (2011) Kanatsu-Shinohara et al. (2008a)
Fgf2	Sertoli cells	Supports/encourages SSC self-renewal	Mouse Rat Rabbit Hamster	Kubota et al. (2004b) Ryu et al. (2005) Kubota et al. (2011) Kanatsu-Shinohara et al. (2008a)
Lif	Peritubular myoid cells	Stimulates proliferation of undifferentiated spermatogonia in conjunction with Gdnf	Mouse	Wang et al. (2014)
Igf-1	Leydig cells	Enhances SCC renewal	Mouse	Kubota et al. (2004b)
Retinoic acid	Sertoli cells? Preleptotene spermatocytes?	Stimulates differentiation of progenitors	Mouse	Dann et al. (2008), Raverdeau et al. (2012), and Tong et al. (2013)
Csf1	Leydig cells, myoid cells	Stimulates self-renewal of SSCs	Mouse	Oatley et al. (2009)

5.5 Tools to Study the SSC Population

Historically, progress in characterizing the SSC population has been marred by several limitations. For instance, the rarity of SSCs within an undifferentiated spermatogonial population in which progenitors are much more abundantly represented, and also the lack of markers available to distinguish spermatogonial subtypes. As a consequence of these difficulties, the field has focused on advancing in vitro culture techniques to provide a sustainable population of cells to study, on further identifying SSC-specific factors expressed both intrinsically and on the surface of the cell, and on formulating novel methodologies to monitor and analyze SSC content within the heterogeneous spermatogonial population. The tools that are currently available for studying the SSC population are discussed below.

5.5.1 Primary Spermatogonial Cultures

Key limitations in studying SSCs are their rarity in the testis, along with the difficulty of directly manipulating these cells in vivo without interfering with the function of other germ cell populations or somatic support cells. As such, primary spermatogonial culture techniques have been continuously refined to provide a platform for producing larger numbers of cells for analysis, and a means for experimental manipulation that directly assesses the role of genes, molecular pathways, and extrinsic signals on self-renewal and maintenance of the SSC population. In refining cell culture techniques, replication of conditions within the stem cell niche is imperative, not only to maintain viability of these cells, but also to prevent entry into a differentiating pathway. As mentioned previously, the somatic support cells of the SSC niche release growth factors to stimulate self-renewal of the SSC population, and as such, a subset of these growth factors have been harnessed for the development of culture media that supports long-term maintenance of SSCs; including Gdnf, and Fgf2.

The assessment of culture conditions for supporting long-term maintenance of SSCs in vitro has been achieved using defined serum free media (Kubota et al. 2004b), to which desired growth factors are incrementally added to establish their effects on the SSC population. Importantly, SSCs are routinely cocultured with SIM mouse embryo-derived thioguanine and ouabain resistant (STO) feeder cells that are known to support culture of several stem cell types (Matsui et al. 1992; Nagano et al. 1998; Paratcha et al. 2003); likely by secreting several growth factors that are component of the respective niches in vivo. Medium that can promote SSC maintenance in vitro is identified by the formation and expansion of colonies of undifferentiated spermatogonia, as well as the ability for a portion of the cells to regenerate spermatogenesis in recipient testes posttransplantation. The addition of Gdnf alone to serum free culture medium has differing outcomes dependent on the strain of mouse from which the primary spermatogonial culture originated. For the spermatogonial population from mice with a DBA/2J genetic background, Gdnf supplementation alone supports colony expansion, and a subset of the population is able to

efficiently engraft in recipient testes following transplantation at various times of the culture period, thereby demonstrating self-renewal of SSCs (Kubota et al. 2004b). However, SSCs from other mouse genetic backgrounds (e.g. C57BL/6) do not thrive in these conditions. In these circumstances, SSC maintenance is achieved with the supplementation of Gdnf in combination with Fgf2 (Kubota et al. 2004b). Conflicting data exists as to whether Fgf2 alone can support in vitro proliferation of SSCs (Kubota et al. 2004b; Takashima et al. 2015). However, in combination with Gdnf, Fgf2 certainly improves long-term culture efficiency for spermatogonia from rabbits (Kubota et al. 2011), hamsters (Kanatsu-Shinohara et al. 2008b) and rats (Ryu et al. 2005).

Although several additional growth factors are known to stimulate proliferation and self-renewal of stem cells from other tissue types, the supplementation of these factors to SSC culture medium has been found to be ineffective or inconsistent. For instance, although Lif is critical for proliferation and maintenance of embryonic stem cell populations in vitro (Murray and Edgar 2001), and further, is known to be produced by the peritubular myoid cells in the testes (Piquet-Pellorce et al. 2000; Dorval-Coiffec et al. 2005); several studies have reported that supplementation of Lif into serum free culture medium has no demonstrable effect on the proliferation of rodent SSCs in vitro (Kubota et al. 2004b; Wang et al. 2014). Contrastingly, SSC proliferation was found to be improved when Lif was used in conjunction with Gdnf (Wang et al. 2014), but again, this is result is controversial (Nagano 2003). Numerous further growth factors have also been analyzed, including stem cell factor, epidermal growth factor, insulin-like growth factor 1 (Igf-1), and Noggin; however, of these factors only Igf-1 improved the maintenance of SSCs (Kubota et al. 2004b) (Table 5.1).

Although in vitro culture techniques for rodent spermatogonia have significantly advanced over the past 20 years, these cultures are still time-restricted; exhibiting a decline in stem cell number after prolonged periods (>6 months) (Kanatsu-Shinohara et al. 2005; Helsel et al. 2017a). Further, success with culture of human spermatogonia in these rodent-optimized conditions is severely limited, and remains a considerable challenge in the field (Zheng et al. 2014; Gassei and Orwig 2016). Together, these issues highlight the need for further refinement of culture conditions; potentially by reassessing the metabolomic requirements of undifferentiated spermatogonia, as well as the differences between rodent and primate models. Such advances are critical if SSC culture is to be considered a component of therapeutic strategies to reverse chemotherapy-induced infertility.

5.5.2 Cell Surface Markers

Clearly, characterization of the undifferentiated spermatogonial subtypes is more easily facilitated if pure populations of SSCs and progenitors can be isolated for analysis. The isolation of live SSC populations from the testes or from primary cultures of undifferentiated spermatogonia using antibody-driven strategies requires the elected “marker” to be expressed on the cell surface, following which magnetic

or fluorescence activated cell sorting techniques (MACS and FACS respectively) can be utilized. Unfortunately, to date, no surface markers have been identified that can unequivocally distinguish SSCs from undifferentiated progenitor spermatogonia. Despite this, a number of cell surface markers have been identified that can be utilized to enrich SSCs in a spermatogonial population when compared to an unselected control. A comprehensive list of cell surface markers that have been characterized for SSC enrichment is provided in Table 5.1, along with their efficiency. Cell adhesion molecules predominate this list, being an attractive area of investigation due to their purported role in "homing" of SSCs to the stem cell niche (Kanatsu-Shinohara et al. 2008a).

The cell adhesion molecules α6 and β1 integrin were the first candidates identified to have some capacity to facilitate enrichment of the SSC population. The expression of both molecules had been detected on the surface of spermatogonia within the undifferentiated population (Shinohara et al. 1999; Ebata et al. 2005); where their function was hypothesized to be the binding of laminin in the basement membrane of seminiferous tubules. Indeed, disruption of β1 integrin expression impaired the capacity of SSCs to regenerate spermatogenesis following transplantation into recipient testes (Kanatsu-Shinohara et al. 2008a); making these factors attractive candidates for SSC-specificity. Despite this, isolation of the $\alpha6^+$ and $\beta1^+$ populations from the testis resulted only in an eight and fourfold enrichment of the SSC population, respectively, when compared to an unselected control population (Shinohara et al. 1999); reflecting the expression of these markers not only in SSCs, but also cells within the progenitor population.

Several other cell adhesion molecules were characterized in the undifferentiated spermatogonial population in the years following the discovery of α6- and β1-integrin, including CD9, epithelial cell adhesion molecule (EpCAM), and Cadherin 1 (Cdh1). Both CD9 and EpCAM were originally classified as surface markers of embryonic stem cells. For the germline, selection of the CD9+ population leads to a sevenfold enrichment for SSCs in rodents (Kanatsu-Shinohara et al. 2004a); however, this is limited by the fact that CD9 is also expressed by differentiating germ cells and somatic cells. Similarly, EpCAM expression extends beyond SSCs to progenitors. As such, EpCAM enriched populations provide only a threefold increase in SSC content, as determined by spermatogonial transplantation analyses (Kanatsu-Shinohara et al. 2011). Isolation of the Cdh1+ population produces a similar result, again as a consequence of universal staining across the entirety of the undifferentiated spermatogonial population (Tokuda et al. 2007).

In order to increase enrichment efficiency, cell adhesion surface markers are regularly used in conjunction with other available surface antigens, or fluorescent assays. For example, isolation of $\alpha6^+$ testis cells that are also c-Kit- and MHC class I- significantly improves repopulation efficiency following transplantation (Kubota et al. 2003); with c-Kit being a marker of germ cell differentiation that is evident from the $A_{aligned}$ stage onwards (Schrans-Stassen et al. 1999), and MHC-I a marker thought to be present on the surface of almost all nucleated cells, however, absent from the spermatogonial population (Glynn 1988). Alternatively, the utilization Cdh1 labeling in conjunction with a fluorescent assay that monitors aldehyde

dehydrogenase (Aldh) activity allows for isolation of the Cdh1+, Aldh1− spermatogonial population that is enriched for SSC concentration when compared to the Cdh1+ population alone (Kanatsu-Shinohara et al. 2013). Although such multiparameter techniques facilitate the isolation of a population that is enriched for SSCs, such isolation strategies are associated with greater complexity and increased cell loss from testis preparations.

To date, Thy1; a phosphatidylinositol-anchored surface antigen that is known to be expressed in a hematopoietic (Spangrude et al. 1988), mesenchymal (Jiang et al. 2002) and embryonic (Ling and Neben 1997) stem cells; has arguably been the most valuable surface marker characterized as a single-parameter for SSC enrichment. The Thy1+ fraction isolated from mouse testes is enriched for SSC number by 30-fold compared to the nonselected total testis fraction of adult mice (Kubota et al. 2004a). As a consequence of the clearly superior SSC enrichment capacity of Thy1+ when compared to the other surface markers discussed, it is not surprising that this surface antigen is commonly employed in current SSC research, particularly for enriching SSC populations to generate primary spermatogonial cultures (Oatley et al. 2009; Helsel et al. 2017a). Additionally, the Thy1 enriched spermatogonial population has proved to be valuable for identifying intrinsic factors involved in regulating SSC maintenance and self-renewal (Oatley et al. 2007). Despite the value of Thy1 as an SSC surface marker, it still does not facilitate isolation of a pure SSC population; with the estimation of SSC content in the Thy1+ population being 1 in 15 cells (Kubota et al. 2003).

In the search for a SSC-specific marker, it is not surprising that receptors for growth factors produced by niche support cells to stimulate self-renewal of SSCs have been attractive candidates. As mentioned previously, Gfrα1 is part of the binding receptor complex for Gdnf (He et al. 2007); with Gdnf stimulating self-renewal of SSCs in vitro (Kubota et al. 2004b). Unfortunately, however, SSC content from Gfrα1+ populations of spermatogonia is only slightly enriched compared to that of unselected control cells in the pup testis, and in fact, SSC content is unchanged from the unselected control population in adult testes (Ebata et al. 2005); making this marker an undesirable choice for isolating pure SSC populations. Upon further investigation, it was found that Gfrα1 expression was seemingly uniform across the entire population of type-A spermatogonia in both mouse (Ebata et al. 2005; Grasso et al. 2012) and human testis (Grisanti et al. 2009; He et al. 2010), thus explaining the lack of SSC enrichment achieved by isolating cell fraction using this surface marker.

Although the capacity for the aforementioned surface markers to enrich the SSC population has been primarily focused on rodent species, the expression of these surface antigens appears to be relatively conserved between rodents and primates, with putative SSCs in rhesus macaque testis being $Thy1^{+}$, $Gfr\alpha1^{+}$ and c-Kit^{-} (Hermann et al. 2009). Additionally, some putative human SSC markers, such as stage-specific embryonic antigen 4 (Ssea4), have been identified (Kokkinaki et al. 2011). Despite this, a gap in knowledge clearly still exists in the elucidation of surface markers that are truly SSC-specific. Although this limitation has been somewhat circumvented in rodent species as a consequence of the identification of

intrinsic factors solely expressed in the SSC population, as well as the capacity to generate transgenic lines that express fluorescent reporters based on these intrinsic factors (discussed below); such strategies are clearly not translatable to the study of human SSC populations, and remain difficult in the majority of other mammalian species. As such, further investigation is required to establish surface markers that are SSC-specific, and exhibit expression that is conserved across a range of mammalian species.

5.5.3 Spermatogonial Transplantation

The development of a spermatogonial transplantation technique in rodents in 1994 (Brinster and Avarbock 1994; Brinster and Zimmermann 1994) was a turning point in the field of SSC research, as it provided a definitive means by which to determine SSC content within a heterogeneous spermatogonial population, and with which to make direct quantitative comparisons between spermatogonial populations; for example, between unselected spermatogonial populations and those enriched with one of the aforementioned cell surface markers. Indeed, while morphological observation of the spermatogenic lineage within a testis that has been subjected to fixation following in vivo genetic manipulation, or assessment of cell proliferation in response to in vitro manipulation, may provide insight into SSC dynamics, currently, the only unequivocal methodology to quantitatively assess SSC content is to analyze the ability of these cell to regenerate spermatogenic colonies in recipient testes.

Typically, the spermatogonial transplantation technique (Fig. 5.3) involves utilization of a donor mouse that possesses a LacZ transgene in the *Rosa26* locus; driving expression in every cell of the body; including germ cells. The recipient mouse utilized for this procedure must be devoid of endogenous spermatogenesis (so available niches are not occupied by endogenous SSCs); usually achieved via pretreatment with an alkylating chemotherapeutic agent (i.e. busulfan) that eliminates the germ cell population (Brinster 2002). Spermatogonia taken directly from the donor mouse, or from primary spermatogonial cultures established from the donor mouse, are injected into the rete testis of the recipient. Following this surgical procedure, the recipient testes are analyzed 2–5 months later to allow time for donor SSCs to regenerate colonies of persistent spermatogenesis. Clearly, only true SSCs in the donor population can incorporate into the stem cell niche and reestablish continual spermatogenesis. Thus, in knowing the number of cells injected into the recipient testis, determining the relative percentage of SSCs can be achieved by staining the recipient testis with X-gal and counting the number of LacZ expressing donor-derived colonies. The relative "SSC number" can be reported as the number of colonies generated per 10^5 cells injected in order to create standardization among experiments. To assess the "purity" of SSCs in the original suspension, colonization efficiency needs to be taken into consideration (i.e. not all SSCs injected will migrate to a niche and form a colony). Thus, it has been estimated that approximately 5–12% of transferred SSCs initiate colonization of a busulfan treated testis

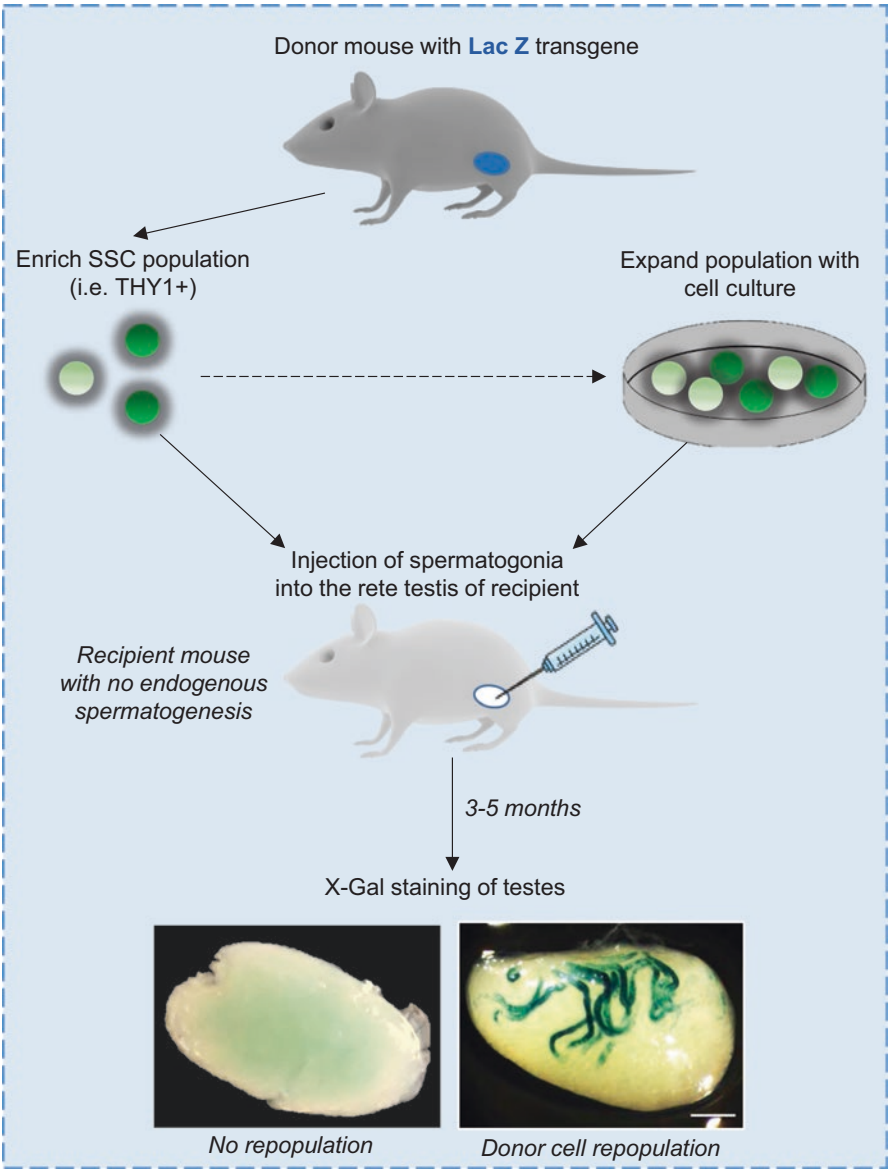

Fig. 5.3 Spermatogonial transplantation is performed using spermatogonia derived from a donor mouse with a Lac Z transgene in the *Rosa26* locus. Spermatogonia retrieved from this donor mouse may either be directly subjected to enrichment strategies (i.e. isolation of the Thy1+ population), or cultured for expansion and/or treatment of spermatogonia. Selected populations are injected into the rete testis of a recipient mouse whose endogenous spermatogenesis has been eliminated using treatment with alkylating chemotherapy. The recipient mouse is subjected to a 3–5 month recovery period to allow donor SSCs to migrate to vacant niches, and begin recolonization of the testes. Donor colonies can be visualized in recipient testis via X-Gal staining, and colonies can be counted to provide quantitative value that reflects SSC content

(Shinohara et al. 2001; Nagano 2003; Ogawa et al. 2003), with each of these colonies arising from a single SSC (Dobrinski et al. 1999).

Certainly, the spermatogonial transplantation technique is immensely useful for assessing the specificity of SSC markers used to enrich the spermatogonial population prior to transplantation, or alternatively, for highlighting changes to the SSC population in response to targeted depletion of factors hypothesized to be important for maintenance of the stem cell state. Further to this, although spermatogonial transplantation is not directly transferrable for the study of human SSC populations, successful autologous (donor and recipient are the same animal) and allogenic (donor and recipient are different animals) transplantation has been achieved in nonhuman primate species (Hermann et al. 2012). These studies potentially pave the way for the development of therapeutic treatments for human cancer survivors whom have been rendered infertile as a consequence of chemotherapy.

5.5.4 Intracellular Markers

With a lack of true SSC-specific surface markers available to isolate live stem cell populations, the field has turned to identifying and characterizing intracellular SSC markers to gain information on this population. This approach largely relies on antibody-driven experiments to make preliminary assessments on the population of interest; usually examining specificity of gene expression amongst the different germ cell populations, and within the undifferentiated spermatogonial population specifically, in fixed and sectioned rodent testes. As antibodies against these intracellular factors cannot be used to isolate the population of interest in the absence of fixation, historically, elucidation of the role of such intracellular spermatogonial markers in the maintenance of the undifferentiated population has been achieved using gene inactivation or knockdown strategies, both in vivo and in vitro. The limitation associated with this approach, in regards to the generation of null mice, is the possibility of causing an embryonic lethal phenotype. Indeed, this has been the case in a number of studies, particularly those assessing the role of pluripotency factors such as Pou5f1 (Nichols et al. 1998); that are expressed across the entire undifferentiated spermatogonial population (Ohbo et al. 2003) and play an important role in preimplantation embryo development. To circumvent this, RNAi-driven knockdown of these intrinsic factors is often conducted in primary cultures prior to spermatogonial transplantation (Dann et al. 2008), effectively demonstrating any involvement in maintenance of the undifferentiated spermatogonial population.

A number of intracellular spermatogonial markers have been identified and characterized in this manner; however, the vast majority of these markers are, again, not SSC-specific, but rather expressed throughout the undifferentiated population (i.e. in A_{single}, A_{paired} and $A_{aligned}$ cells). These factors include Zinc finger and BTB domain containing 16 (Zbtb16; i.e. Plzf) (Buaas et al. 2004; Costoya et al. 2004), Neurogenin3 (Neurog3) (Yoshida et al. 2004, 2006, 2007; Zheng et al. 2009), Nanos2 (Suzuki et al. 2009), Lin28 (Zheng et al. 2009) and Pou5f1 (Dann et al. 2008) (a comprehensive list of intracellular factors is provided in Table 5.3). While

helpful for distinguishing the undifferentiated spermatogonia from other germ cell types in the testes, as well as identifying heterogeneity in this population (Suzuki et al. 2009; Niedenberger et al. 2015), and allowing for study into how the undifferentiated population is maintained, such markers do not provide a direct means to study the SSC population specifically.

Fortunately, recent research has identified two candidates whose expression profiles appear to exhibit a high degree of specificity to the SSC population; Id4 (Oatley et al. 2011b; Chan et al. 2014) and Paired box 7 (Pax7); whose theoretical roles in SSC maintenance and self-renewal will be explored later in this chapter. As mentioned previously, the identification of these SSC-specific factors, particularly Id4, arose from differential expression analyses on Thy1+ enriched spermatogonial populations as compared to Thy1− spermatogonia (Oatley et al. 2009); and further characterization was achieved using the aforementioned antibody-driven and gene knockdown techniques (Oatley et al. 2009). The true value of identifying these intracellular SSC-specific markers is realized when they are utilized for the generation of reporter transgene constructs and subsequent mouse lines, as will be discussed below.

5.5.5 Reporter Transgenes

The utilization of reporter transgenes has allowed for the development of mouse lines in which the spermatogonial population, and more recently the SSC population specifically, can be identified by expression of a fluorescent marker; usually Gfp, or alternatively a colorimetric marker such as LacZ. Reporter transgene constructs have been generated using the pan-undifferentiated markers Pou5f1 (Youn et al. 2013), Nanos3 (Yamaji et al. 2010) Neurog3 (Yoshida et al. 2004) and Sox2 (Arnold et al. 2011), as well as the putative SSC-specific markers Id4 (Chan et al. 2014) and Pax7 (Aloisio et al. 2014).

The advantage of such mouse lines are numerous. Firstly, reporter transgenes make it possible to perform live tissue "whole mount" analyses of testicular tubules, allowing for identification of spermatogonial cell subtypes that are expressing the factor of interest (i.e. single, paired and/or aligned structures) without the disruption that is caused by fixation and sectioning of tissues (Chan et al. 2014). Such analyses provide further clarification as to whether the factor of interest is likely to be a marker of pan-undifferentiated spermatogonia (i.e. expression in single, paired and aligned structures), or potentially a marker that is SSC-specific (i.e. only expressed in a small percentage of the A_{single} population). Such live imaging techniques have also been utilized to create three dimensional reconstruction of the testicular tubules, for instance with a specific focus on the placement of Neurog3-Gfp undifferentiated spermatogonia in relation to surrounding blood vessels (Yoshida et al. 2007).

The development of reporter mouse lines allows for FACS isolation of selected spermatogonial populations, again, circumventing both the requirement for selective factors to be expressed on the cell surface, and the need for antibodies and fixatives. Thus, the selected live spermatogonial population can be retrieved from the

testes, and purity of SSCs in the population (and thus specificity of the intrinsic marker) can easily be assessed via spermatogonial transplantation. For example, transplantation of isolated Gfp+ and Gfp− spermatogonia from in the *Id4-eGfp* mouse line (Chan et al. 2014) was integral in demonstrating the high degree of specificity of this marker for the SSC population; with >90% of regenerative spermatogonia being contained within the Gfp+ population. The capacity for SSC enrichment using this Id4-driven reporter transgene when compared to enrichment values achieved with other reported cell surface markers (Table 5.2), demonstrates the value of using such strategies for the study of the SSC population.

In addition to the aforementioned applications, spermatogonial populations derived from mouse lines containing reporter transgenes that label subsets of the undifferentiated spermatogonial population can be used to establish primary spermatogonial cultures. Such strategies provide the field with a means to rapidly monitor changes in transgene expression (as a function of fluorescence), and thus potential changes to the composition of the spermatogonial population. This application may be particularly useful for monitoring SSC dynamics in response to in vitro genetic manipulation (for instance RNAi knockdown), altered culture environments, and exogenous growth factors. Further, these technologies have provided

Table 5.2 Surface markers utilized for FACS or MACS isolation to enrich SSC populations in adult and pup testis suspensions

Surface marker	Expression/specificity	Colonization compared to unselected control (colonies per 10^5 cells)	Reference
Gfrα1	SSCs and progenitors	0.13 × *Adult* (7.12) 2.5 × *Pup* (45)	Ebata et al. (2005)
β1 integrin	SSCs and progenitors	4 × *Adult* (30)	Shinohara et al. (1999)
a6 integrin	SSCs and progenitors	8 × *Adult* (55)	Shinohara et al. (1999)
CD9	SSCs and some differentiated spermatogonia	7 × *Adult* (55)	Kanatsu-Shinohara et al. (2004b)
EpCAM	All spermatogonia; however, more strongly expressed in progenitors	3 × *Adult* (6)	Kanatsu-Shinohara et al. (2011)
Thy1	SSCs and progenitors	30 × *Adult* (48.1) 5 × *Pup* (69.6)	Kubota et al. (2004a)
Cdh1	SSCs and progenitors	N/A *Adult* (34.7 compared to 0 in CDH1-population)	Tokuda et al. (2007)

Enrichment efficiency is reported as a function of colonization of recipient testes following spermatogonial transplantation, as compared to an unselected control

a gateway for performing large-scale comparative analyses between putative SSC and progenitor populations; i.e. differential RNA-seq analysis (Chan et al. 2014); to further explore expression profiles that may be unique to SSCs, to identify how putative SSC-specific factors may be driving maintenance and self-renewal of this population, and which factors are integral for initiating the commitment to progenitor formation.

5.5.6 Lineage Tracing

Lineage tracing is a technique that provides an alternative to spermatogonial transplantation for assessing the specificity of a selected marker for the SSC population. Using this methodology, putative SSCs are permanently labeled (i.e. with LacZ or Gfp) by an inducible reporter transgene, and all daughter cells arising from this clone retain the label, allowing cell lineage to be traced back to the original cell. For example, using a tamoxifen-induced Cre, Sun et al. (2015) demonstrated that Id4+ cells in the adult testis gave rise to paired and aligned structures 5 days post-tamoxifen treatment, and continued to produce labeled clones at all stages of spermatogenesis at both 5 and 13 months post-tamoxifen injection. This strategy was also adopted by Aloisio et al. (2014) to demonstrate that Pax7+ spermatogonia undergo self-renewal, in addition to generating progenitors that experience clonal expansion, and eventually produce spermatozoa. The rationale behind this technique is that only true SSCs will continue to produce clones several months after tamoxifen-induced labeling, whereas progenitor spermatogonia, for instance, would only produce downstream labeled cells for the rounds of spermatogenesis that immediately follow tamoxifen treatment. Importantly, lineage tracing can also be utilized to track formation of the founder SSC population from prospermatogonia (Aloisio et al. 2014; Sun et al. 2015); providing a means to investigate this period in development that remains relatively elusive.

The advantage of lineage tracing above that of spermatogonial transplantation is the ability to monitor stem cell dynamics within physiological context (i.e. in steady-state conditions, without disruption to the population). Despite this, there are limitations to this technique in that, unlike transplantation, lineage tracing does not provide a quantitative assessment of SSC content, but simply depicts that a portion of the selected population does possess stem cell capacity. As such, lineage tracing is not particularly useful for direct quantitative comparisons between populations of spermatogonia. Additionally, the use of tamoxifen-induced Cre system to induce expression of these reporter transgenes creates concerns regarding the disruption of normal steady-state conditions in the testes, as tamoxifen has purported endocrine disrupting capacity (Yu et al. 2014). A diagrammatic comparison between lineage tracing and spermatogonial transplantation as techniques for assessing the SSC population is provided in Fig. 5.4, with a particular focus on the advantages and disadvantages of each technology.

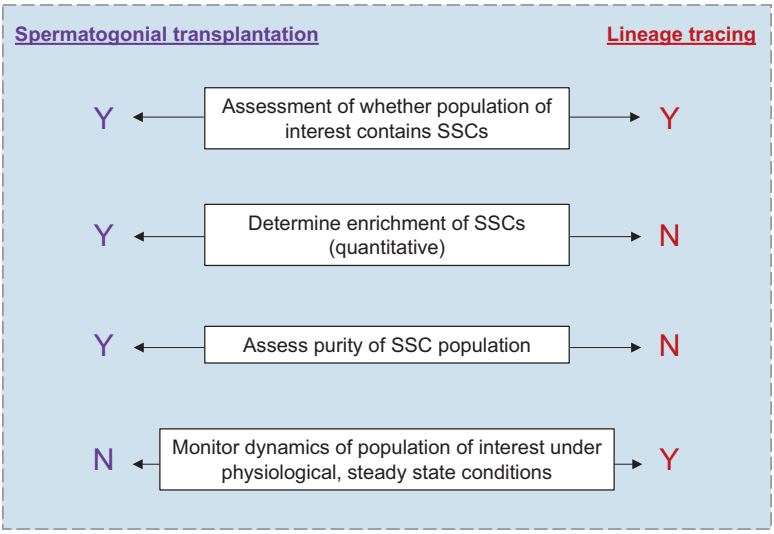

Fig. 5.4 Comparison of spermatogonial transplantation and lineage tracing strategies for assessing the SSC population

5.6 Molecular Regulation of SSC Maintenance; Transcription Factors, miRNAs, and Translational Regulators

Using the experimental techniques that have been discussed above, several intrinsic regulators, including transcription factors, miRNAs, and other posttranscriptional effectors have been identified that are involved in orchestrating SSC self-renewal, or alternatively, in maintenance of the undifferentiated state in both SSCs and progenitors. Below, we explore these factors in terms of their purported influence on SSC dynamics, and any established relationship between these factors and the extrinsic growth factors listed in Table 5.1. Key intrinsic regulatory molecules that are discussed below and their theoretical functions are summarized in Table 5.3, and a schematic of our current understanding of the processes controlling SSC renewal, and maintenance of the undifferentiated state in both SSCs and progenitors is provided in Fig. 5.5.

5.6.1 Transcription Factors

Not surprisingly, transcription factors appear to be at the spearhead of maintaining the stem cell state; with knockdown of a multitude of these factors resulting in significantly impaired SSC maintenance, stimulating loss of the undifferentiated population to differentiation, and in many cases resulting in infertility. Below we examine

Table 5.3 Intrinsic factors involved in regulation of undifferentiated spermatogonial populations

Regulatory factor	Classification	Effect on undifferentiated population	Reference
Bcl6b	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Oatley et al. (2006, 2007)
Etv5	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Oatley et al. (2006, 2007)
Lhx1	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Oatley et al. (2006, 2007)
Pou3f1	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Wu et al. (2011)
Foxo1	Gdnf-regulated transcription factor	May be involved in SSC maintenance and self-renewal	Goertz et al. (2011)
Plzf/Zbtb16	Zinc finger protein/transcription factor	Required for maintenance of the undifferentiated population	Buaas et al. (2004), Costoya et al. (2004), and Dann et al. (2008)
Taf4b	TATA binding protein	Required for maintenance of the undifferentiated population	Lovasco et al. (2015)
Oct4	Pluripotency transcription factor	Required for maintenance of the undifferentiated population	Dann et al. (2008)
Sox2	Gdnf-regulated, pluripotency transcription factor	Involved in maintaining the undifferentiated population?	Arnold et al. (2011) and Oatley et al. (2007)
Sall4	Pluripotency transcription factor	Required for maintenance of the undifferentiated population	Gassei and Orwig (2013)
Rb1	Transcription factor	Involved in formation of SSC pool and SSC maintenance	Hu et al. (2013) and Yang et al. (2013b)
Id4	Gdnf-regulated, SSC-specific HLH transcription factor	Required for SSC renewal/maintenance	Oatley et al. (2011b) and Chan et al. (2014)
Pax7	Putative SSC-specific transcription factor	Involved in stem cell renewal/maintenance?	Aloisio et al. (2014)
Sox3	"High mobility group" transcription factor	Required for progenitor differentiation	Raverot et al. (2005)
Stat3	Transcription factor	Required for progenitor differentiation	Kaucher et al. (2012) and Oatley et al. 2010)

(continued)

Table 5.3 (continued)

Regulatory factor	Classification	Effect on undifferentiated population	Reference
Ngn3	HLH transcription factor, Gdnf-regulated	Required for progenitor differentiation	Kaucher et al. (2012)
Sohlh1/2	HLH transcription factor	Required for progenitor differentiation	Ballow et al. (2006) and Suzuki et al. (2012)
miR21	microRNA	Involved in maintaining an undifferentiated state	Niu et al. (2011)
miR221, miR222	microRNA	Involved in maintaining an undifferentiated state	Yang et al. (2013b)
miR146	microRNA	Modulates differentiation in response to retinoic acid	Huszar and Payne (2013)
miR20, miR106a	microRNA	Involved in maintaining an undifferentiated state	He et al. (2013)
Nanos2	Translational repressor	Acts with mRNPS to repress translation of differentiation-related mRNAs	Zhou et al. (2015b)

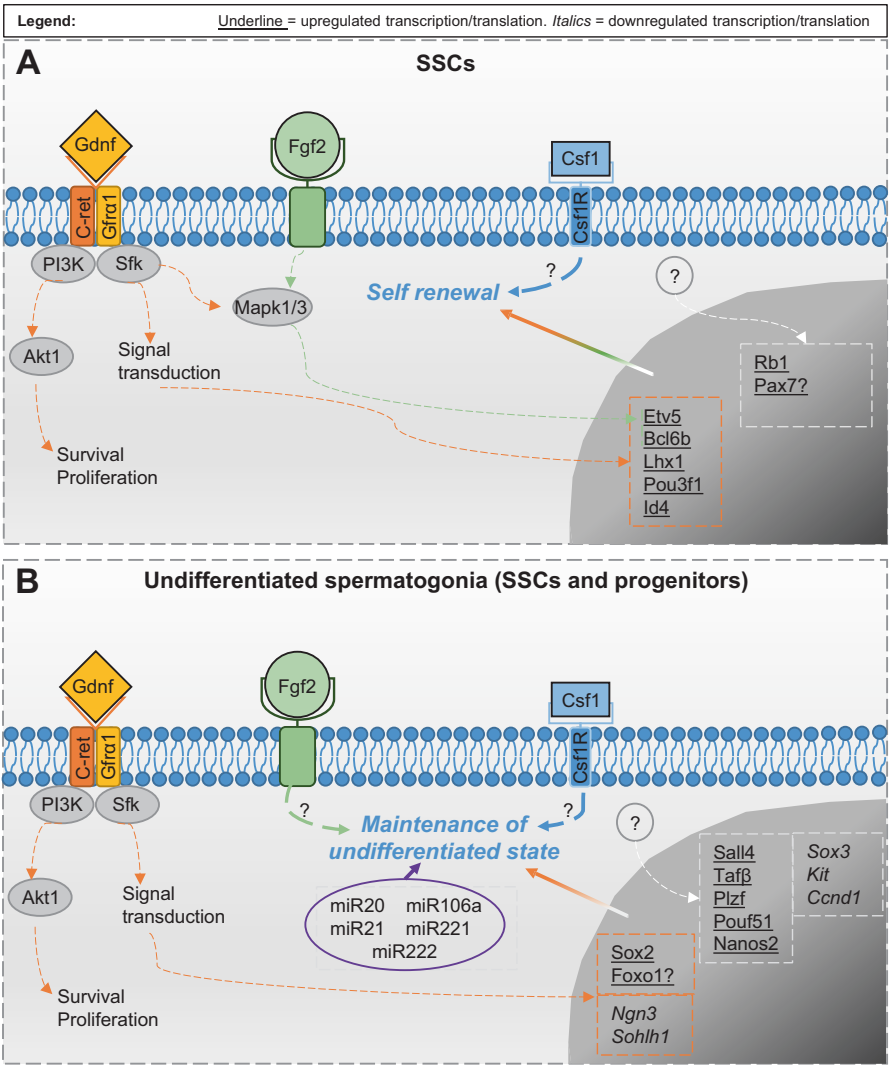

Fig. 5.5 Diagrammatic representation of factors involved in self-renewal of SSCs (**a**), and maintaining an undifferentiated state in SSCs and progenitors (**b**). (**a**) Gdnf signaling via the c-Ret/Gfrα1 receptor complex has been directly connected with increased self-renewal of SSCs in vitro. Etv5, Bcl6b, Lhx1, Pou3f1 and Id4 experience prominent upregulated expression in response to Gdnf signaling, purportedly via a PI3K/SFK-driven signal cascade. Further, Etv5 and Bcl6b are upregulated by an Fgf2-driven Mapk signaling cascade. (**b**) A number of intracellular factors have been identified that are expressed in both SSC and progenitor populations, whose knockdown results in loss of these populations to differentiation. While genes such as Sox2 and members of the Foxo gene family are upregulated by Gdnf, differentiation-driving factors such as Ngn3 and Sohlh1 are downregulated in response to Gdnf signaling pathways. A number of Gdnf-independent genes are also upregulated in response to unknown signaling pathways to maintain the undifferentiated state, while Sox3, Kit, and Ccnd1; again responsible for driving differentiation of spermatogonia, are downregulated in response to unknown external cues. Further, a number of miRNAs are known to be involved in maintaining the population of undifferentiated spermatogonia (*purple circle*), via the degradation of mRNAs that would normally drive differentiation in these cells

transcription factors known to be responsive to growth factor signals from the SSC niche (particularly Gdnf) that stimulate self-renewal of SSCs, transcription factors that appear to influence SSC function independently of Gdnf, factors that maintain pluripotency in embryonic stem cells that also play a role in SSC maintenance, and helix-loop-helix (HLH) factors that have recently emerged as key regulators of the SSC population. We also focus specifically on the putative SSC-specific transcription factors Id4 and Pax7, and their potential roles as "master regulators" of the stem cell state.

5.6.1.1 Gdnf-Dependent

When considering the propensity for growth factors such as Gdnf to stimulate self-renewal and proliferation of SSCs in vitro, it is not surprising that characterization of the transcription factors and signaling pathways that are activated within spermatogonia in response to these growth factors have been a primary focus of investigation. Pioneering studies in 2006 used microarray analyses to identify factors in Thy1+ spermatogonial cultures that were highly influenced by the addition and removal of Gdnf (Oatley et al. 2006). Transcription factors that were identified to be Gdnf responsive included B cell CLL/lymphoma 6 member B (Bcl6b), Ets variant 5 (Etv5), LIM homeobox 1 (Lhx1), Sox2, and Id4 (Oatley et al. 2006, 2007), and in later studies, Pou3f1 (Wu et al. 2011). The role of Bcl6b; the most highly upregulated Gdnf-responsive gene, was demonstrated using RNAi and spermatogonial transplantation. Specifically, following knockdown of Bcl6b, proliferation of spermatogonia in culture was curtailed; and SSC content following transplantation was found to be reduced by >8-fold (Oatley et al. 2006). Further, Bcl6b null mice experienced an age-related loss of fertility characterized by a Sertoli cell only phenotype (Oatley et al. 2006) in accordance with the first rounds of spermatogenesis occurring independently of the SSC population (Yoshida et al. 2004, 2006), while spermatogenesis in adulthood was stunted by the inability for SSCs to self-renew. Subsequent studies demonstrated that knockdown of Etv5, Lhx1, and Pou3f1 has an equivalent effect on proliferation of spermatogonial colonies in vitro, and SSC number following transplantation (Oatley et al. 2007; Wu et al. 2011). Further to these findings, overexpression of Etv5 and Bcl6b has been found to drive rapid self-renewal/proliferation in vitro, and in the case of Bcl6b, overexpression results in the formation of germ cell tumors following transplantation; presumably as a consequence of excessive self-renewal (Ishii et al. 2012). Cumulatively, these data suggest a key role for the Gdnf responsive genes Bcl6b, Etv5, Lhx1, and Pou3f1 in driving self-renewal of SSCs.

Transduction of the Gdnf signal upon binding with its receptor to influence transcription of these target factors has been found to be reliant on Src family kinase (Sfk) signaling downstream of the c-Ret component of the Gdnf receptor (Oatley et al. 2007). Etv5 expression appears to be directly influenced by this Gdnf signaling pathway, with Etv5 itself then stimulating downstream expression of Bcl6b and Lhx1 (Wu et al. 2011). In analyzing the genes targeted by Etv5, Bcl6b, and Pou3f1 using microarray analyses, surprisingly little overlap was found to exist; however,

genes commonly targeted were those involved in cellular proliferation and self-renewal, such as Brachyury (T) (Wu et al. 2011).

Although the Gdnf-Sfk pathway is thought to be directly involved in regulation of the aforementioned transcription factors, the PI3K/Akt pathway that is correspondingly effected by Gdnf-receptor binding has also been demonstrated to be important for SSC survival; with its inhibition resulting in apoptosis (Oatley et al. 2007). Further, more recent studies by Goertz et al. (2011) have identified this Gdnf-driven PI3K/Akt pathway as a regulator of Foxo1; an additional transcription factor that may be involved in SSC maintenance, with c-Ret itself being a potential gene target of this factor. Indeed, combined deficiency of the Foxo family of genes results in impaired fertility and loss of the undifferentiated population (Goertz et al. 2011).

Interestingly, not only has Gdnf signaling been shown to upregulate expression of genes involved in SSC maintenance and renewal, but also to downregulate genes that are required for spermatogonial differentiation. As such, the microarray study performed by Oatley et al. in 2006 identified Neurog3 expression to be significantly downregulated in response to Gdnf exposure; with more recent studies demonstrating that a loss of Neurog3 expression is associated with an inability for spermatogonia to differentiate (Kaucher et al. 2012). Further investigation revealed that Neurog3 is required for "Signal transducer and activator of transcription 3" (Stat3)-driven differentiation of spermatogonia as a consequence of direct binding of Stat3 to the Neurog3 promoter/enhancer (Kaucher et al. 2012).

Although literature on growth factor-regulated transcription factors in the undifferentiated spermatogonial population has been primarily focused on Gdnf, it should be noted that recent publications have also investigated Fgf2-responsive genes. Interestingly, two primary candidates that experienced upregulated expression in response to Fgf2 were also Gdnf responsive genes; namely Etv5 and Bcl6B (Ishii et al. 2012). The Fgf2-initiated response was reported to act via a Mapk1/3 signaling pathway, and dysregulation of this pathway prior to spermatogonial transplantation resulted in the formation of germ cell tumors; again supporting a role for these Gdnf-responsive transcription factors in driving self-renewal of the SSC population (Ishii et al. 2012).

5.6.1.2 Gdnf-Independent

Despite the integral nature of Gdnf signaling, a number of transcription factors that appear to be involved in SSC maintenance have been characterized that act independently of this growth factor; including Plzf, TATA-Box Binding Protein Associated Factor 4b (Taf4b) and retinoblastoma protein (Rb1) (Oatley et al. 2006); likely indicating a gap in knowledge surrounding extrinsic regulatory factors within the SSC niche. The transcriptional repressor Plzf was the first identified intrinsic regulator of undifferentiated spermatogonia and was described in 2004 (Buaas et al. 2004; Costoya et al. 2004). Plzf is known to be involved in regulation of cell cycle progression in other cell types, including hematopoietic stem cells where it appears to be involved in the G1 to S phase transition (Vincent-Fabert et al. 2016). In the testis, Plzf does not exhibit SSC-specific expression; rather, its expression can be identified throughout the undifferentiated spermatogonial population (Costoya et al.

2004). Although not SSC-specific, expression of Plzf is indispensable for maintenance of the SSC population. Plzf knockout mice exhibit sub-fertility and a diminished spermatogonial population, and their germline cells cannot colonize recipient testes upon transplantation (Buaas et al. 2004; Costoya et al. 2004). Regulation of Plzf expression is thought to occur via a phosphatase and tensin homolog (Pten) signaling cascade (Zhou et al. 2015b); and its potential modes-of-action to maintain the undifferentiated state include repression of genes that have been implicated in spermatogonial differentiation, including c-Kit (Filipponi et al. 2007) Cyclin D1 (Ccnd1) (Costoya et al. 2004), and mammalian target of rapamycin complex 1 (mTorc1) (Hobbs et al. 2010). Conversely, expression of Plzf is purportedly downregulated in response to retinoic acid; an event that is likely required to allow for differentiation to occur in progenitor spermatogonia (Dann et al. 2008). Interestingly, knockdown of a component of the TFIID general transcription factor complex, Taf4b, exhibits a similar phenotype to that seen with Plzf in that differentiation is favored over maintenance of the undifferentiated population (Lovasco et al. 2015). Again, however, the extrinsic signals controlling Taf4b expression are unknown, and its expression in undifferentiated spermatogonia is not altered by the presence/absence of Gdnf (Oatley et al. 2006, 2007).

Another factor involved in regulating transcription to maintain the SSC population, independently of Gdnf (Oatley et al. 2006), is Rb1. Like Plzf, Rb1 is known to be a key cell cycle regulator (Cobrinik 2005). In male mice with Rb1 germline inactivation, progressive germline loss is visible from 2 months of age (Hu et al. 2013). Rb1 appears to not only be involved in maintenance of the SSC population; as can be observed as a consequence of reduced testis-colonization following siRNA knockdown and spermatogonial transplantation of cultured spermatogonia; but also in formation of the SSC pool in neonatal development upon the prospermatogonial transition (Yang et al. 2013b). Thus, although the first (and possibly second) round(s) of spermatogenesis occur [a process known to be independent of the SSC population (Yoshida et al. 2004, 2006)], germline cells are progressively lost in mice with Rb1 inactivation, suggesting that the SSC pool was not formed (Yang et al. 2013b). Interestingly, spermatogonial cells with diminished Rb1 expression demonstrate increased tumorigenic properties; invading the basement membrane of the testis posttransplantation; potentially symbolizing a loss of cell cycle control resulting in dysregulated proliferation/self-renewal (Yang et al. 2013b).

5.6.1.3 Pluripotency Transcription Factors

Similar to Plzf and Taf4b, Pou5f1 is a Gdnf-independent transcription factor involved in maintaining the undifferentiated SSC population (Oatley et al. 2006). Pou5f1, Sox2 [Gdnf-dependent (Oatley et al. 2006)] and Spalt-like transcription factor 4 (Sall4), were originally characterized as a genes required for maintaining pluripotency in embryonic stem cells. Despite this, these factors are also known to be expressed in undifferentiated spermatogonia; a unipotent cell type (in physiological conditions). Pou5f1 is a homeobox transcription factor that is widely indispensable for stem cell activity. Pou5f1 knockout in mice causes an embryonic lethal phenotype, as the inner cell mass of the blastocyst does not retain pluripotency

(Nichols et al. 1998). Similarly, knockdown of Sox2 and Sall4 in ES cells results in a loss of pluripotency, purportedly due to the necessity of these factors for maintenance of the required levels of Pouf51 expression (Zhang et al. 2006; Masui et al. 2007).

In the germline, Pou5f1 is expressed throughout the undifferentiated spermatogonial population (Ohbo et al. 2003). Conflicting data exists as to the role of Pouf51 in SSC function, with one study reporting that knockdown of Pou5f1 in spermatogonial cultures resulted in significantly impaired colonization following transplantation (Dann et al. 2008), while another reported no detriment to SSC maintenance (Wu et al. 2010). Despite this, any activity exerted on the undifferentiated spermatogonia population by Pou5f1 appears to be via direct interaction with Sox2 (Takashima et al. 2013); as is the case within embryonic stem cells. In Sox2-reporter mouse lines, Sox2 expression can be identified within A_{single} spermatogonia (Arnold et al. 2011); however, expression cannot be identified using immunohistochemistry techniques, suggesting that this transcription factor is either not translated, or that protein levels are very low (Arnold et al. 2011). Despite this, like Pou5f1, Sox2 has been shown to be expressed by at least a subset of the SSC population; as demonstrated by lineage tracing analyses (Arnold et al. 2011). Finally, Sall4 expression is detectable across the entirety of the undifferentiated spermatogonial population (Gassei and Orwig 2013), with knockdown influencing maintenance of the SSC population. In addition to its potential role in interacting with co-expressed pluripotency factors Pou5f1 and Sox2, Sall4 has also been found to physically interact with Plzf (Hobbs et al. 2012), and target a large number of genes (>2500) in the undifferentiated spermatogonial population (Lovelace et al. 2016).

Interestingly, although SSCs express a suite of pluripotency genes, they themselves do not possess capacity to revert to pluripotency in vivo (Takashima et al. 2013). Theoretically, this may be due to absence of expression of an additional pluripotency factor, Nanog, in the SSC population (Oatley and Brinster 2008). Indeed, Nanog expression is directly related to self-renewal of embryonic stem cells, and is thought to act alongside Pou5f1 to control a myriad of pathways responsible for governing a pluripotent state (Loh et al. 2006).

5.6.1.4 HLH TFs

One family of transcription factors that have recently come to light as key players in SSC maintenance and renewal, as well as in spermatogonial differentiation, are those of the HLH family; which includes Id4, Neurog3, and spermatogenesis and oogenesis specific basic helix-loop-helix transcription factor (Sohlh1). As eluded to previously, Id4 is particularly interesting as is it one of only two identified intrinsic factors whose expression appears to exist within the SSC population specifically. While Id4 is involved in maintenance and self-renewal of SSCs, contrastingly, Neurog3 and Sohlh1 are required for differentiation.

As previously mentioned, Neurog3 expression is negatively regulated by Gdnf (Oatley et al. 2006), and is associated with Stat3-driven differentiation (Kaucher et al. 2012). The “high mobility group” transcription factor Sox3 has also been found to interact with Neurog3, with Sox3 expression being analogous to that of

Neurog3; i.e. expressed in A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia. As such, Sox3 deletion results in infertility as a consequence of complete germline loss from postnatal day 10; with testes retaining only Sertoli cells and undifferentiated spermatogonia (Raverot et al. 2005). Interestingly, downregulation of Sox3 leads to elevated levels of Pou5f1 expression (Raverot et al. 2005), demonstrating the highly regulated balance between maintenance and differentiation of the undifferentiated spermatogonial population.

Similar to Neurog3; Sohlh1 and 2 are found to be uniformly expressed across the type A spermatogonial population. Sohlh1 expression is driven by Bmp4/Smad signaling (Li et al. 2014) and also appears to be influenced by Gdnf (Grasso et al. 2012). In accordance with their roles in driving spermatogonial differentiation, loss of Sohlh1/2 expression results in infertility accompanied by a reduction in expression of other key genes involved in differentiation (including Neurog3, c-Kit and Sox3), and increased expression of factors involved in stem cell maintenance (Ballow et al. 2006; Suzuki et al. 2012). Both Sohlh1 and 2 individual knockouts show similar phenotypes, namely, these adult mice possess only Sertoli and spermatogonial cells in their testes (Suzuki et al. 2012).

5.6.1.5 SSC-Specific Transcription Factors

As previously mentioned, a milestone has been achieved in recent years in the identification of two factors that are potentially SSC-specific; Id4 and Pax7. Although a number of factors, discussed above, have been reported to be important for maintenance of the SSC population, the expansive expression of these genes between the SSC and progenitor populations suggest that there must be a gap-in-knowledge in factors regulating SSC dynamics. Indeed, the expression of factors such as Id4 and Pax7 in SSC but not progenitor populations makes them strong candidates for orchestrating self-renewal; a characteristic that closely related undifferentiated progenitors do not share.

The inhibitor of DNA binding proteins are transcriptional repressors, traditionally expressed in undifferentiated populations of cells. While Id2 and 3 have been identified in Sertoli cells, and Id1 within spermatocytes, Id4 expression has been established to exist solely within populations of type A spermatogonia within the testis (Oatley et al. 2011b). In conjunction with the revised A_{single} model of spermatogonial maintenance (Helsel et al. 2017b; Lord and Oatley 2017), Id4 is expressed heterogeneously in the A_{single} pool of cells; within less than 10% of the population (Oatley et al. 2011b; Chan et al. 2014). The frequency of Id4 expressing cells peaks in the testis during neonatal development, then decreases significantly in adulthood (Chan et al. 2014), in-line with the developmental kinetics of the SSC population. Importantly, male Id4 null mice possess impaired spermatogenesis that is exacerbated with age; a hallmark characteristic of impaired maintenance of the SSC population. Specifically, seminiferous tubules within the testes of these mice attain a Sertoli-cell-only phenotype (Oatley et al. 2011b). Further, Id4 knockdown in spermatogonial cultures, followed by transplantation, depicts impaired maintenance of the SSC pool (Oatley et al. 2011b). As mentioned earlier

in this chapter, the generation of an *Id4-eGfp* reporter mouse line has allowed for further confirmation that expression of this factor is highly specific to the SSC population; with FACS sorted Id4-eGfp+ spermatogonia derived from primary cultures encompassing >90% of the regenerative spermatogonial population in mice (Chan et al. 2014). Further, it was determined using a limiting dilution transplantation approach that the spermatogonia in the testis expressing the highest levels of Id4 (denoted the Id4-eGfp "bright" cells) represent a population in which 1 in every 0.94 cells is an SSC; i.e. this is an essentially pure SSC pool (Helsel et al. 2017b).

Regarding functional roles in SSCs; expression of Id4 is upregulated by Gdnf signaling and may act to repress expression of genes that drive progenitor formation, such as Neurog3 (Oatley et al. 2009; Helsel et al. 2017b). Additionally, it is plausible that Id4 expression influences stem cell state via regulation of the cell cycle. Quiescence or "slow cycling" is a common property of stem cells; for instance, slow cycling HSCs have the highest long-term stem cell potential, while the more rapidly cycling cells have little to no stem cell capacity (Fuchs 2009). Indeed, it has recently been reported that Id4-eGfp "bright" spermatogonia rarely exhibit EdU incorporation in vivo (Zhang et al. 2016). Further, Id4 has been found to directly interact with key cell cycle regulator Rb1 in the undifferentiated spermatogonial population (Yang et al. 2013b); an interaction that has been implicated to influence cell cycle regulation in other cell types (Zebedee and Hara 2001). Certainly, it will be important to continue investigation into Id4 modes-of-action in rodent SSC populations, as well as establish any conserved expression of this factor amongst other mammalian species including humans.

More recently, Pax7 has been characterized as a transcription factor with an expression profile purportedly specific for SSCs (Aloisio et al. 2014). As described for Id4, $Pax7^{+}$ cells were found to be abundant in neonatal testis; however, they made up only a small portion of the A_{single} cells in the adult testis. Lineage-tracing studies demonstrated that the $Pax7^{+}$ spermatogonia in the adult testis could undergo both self-renewal and produce progenitors to fuel spermatogenesis, suggesting that this factor is expressed in at least a subset of the SSC population. Interestingly, the Pax7 expressing population of spermatogonia has been found to persist following chemotherapy/radiotherapy treatment, and subsequently to be capable of repopulating the testis to restore fertility (Aloisio et al. 2014). Although Pax7 is a high-priority contender for further analysis, the consequences of Pax7 deficiency on the SSC population are yet to be elucidated, and quantitative comparisons of SSC content between Pax7+ and Pax7− populations using transplantation analyses have not yet been conducted. Thus, it is too early to confirm whether this factor is truly SSC-specific. Importantly, however, expression of Pax7 in the undifferentiated spermatogonial population does appear to be relatively phylogenetically conserved, with expression detected in testis sections from domestic animals, nonhuman primates and humans (Aloisio et al. 2014). As such, continued research in to the extrinsic molecules and signaling pathways that regulate Pax7 expression certainly have clinical implications.

5.6.2 miRNAs

Although the role of transcription factors in maintaining the SSC population is clearly integral, regulation of stem cell dynamics and fate decision is also known to occur at the posttranscriptional level; for instance, via transcript degradation or inhibition of translation by miRNAs. The generation of miRNAs within germ cells has been demonstrated to be important for the maintenance of spermatogenesis, as the ablation of DICER and DROSHA; processing enzymes critical for the formation of mature miRNAs, results in infertility as a consequence of azoospermia (Wu et al. 2012). A myriad of different miRNAs are enriched in undifferentiated spermatogonia specifically (Wang and Xu 2015), including miR20 and miR106a (He et al. 2013). Following spermatogonial differentiation (induced by retinoic acid), expression of these miRNAs is greatly reduced. Both in vivo and in vitro analyses utilizing miRNA mimetics and inhibitors suggest a role for miR20 and miR106a in regulation of SSC proliferation/renewal, purportedly via direct interaction with Stat3 and Ccnd1 at the posttranscriptional level (He et al. 2013). Similarly, miR21 expression has been found to be enriched in the Thy1+ fraction of testis cells, with inhibition resulting in reduced colonization of recipient testes following transplantation, and elevated levels of apoptosis (Niu et al. 2011). Micro RNAs 221 and 222 also appear to be involved in maintaining spermatogonia in an undifferentiated state, as inhibition of these miRNAs results in transition of spermatogonia from a c-Kit- to c-Kit+ state (Yang et al. 2013a); a hallmark of the transition from undifferentiated to differentiating spermatogonia. Additionally, in contrast to the aforementioned miRNAs, miR146 appears to be involved in modulating differentiation rather than self-renewal of SSCs, specifically in response to retinoic acid signaling (Huszar and Payne 2013).

5.6.3 Translational Regulators

Finally, in addition to the aforementioned transcription factors and miRNAs involved in intrinsic control of SSC maintenance and self-renewal, current research has identified a further layer of regulation of these processes at the posttranscriptional level. Specifically, Nanos2, an evolutionarily conserved RNA-binding protein whose expression is enriched in the undifferentiated spermatogonial population (A_{single} and A_{paired}), has been found to directly interact with messenger ribonucleoprotein (mRNP) complexes to form a "buffering system" that controls fate decision. In the presence of Nanos2, mRNPs condense to trap differentiation-related mRNAs such as Solhlh2, and signal transducers such as mTOR; preventing their translation and keeping SSCs and progenitors in an undifferentiated state. Alternatively, when Nanos2 expression is reduced, mRNP granules breakdown, releasing the entrapped differentiation-driving mRNAs and allowing for their expression (Zhou et al. 2015a). The key role for Nanos2 in maintenance of SSCs was demonstrated by knockout of this gene in the spermatogonial population, which caused stem-cell depletion. Contrastingly, overexpression of Nanos2 in

SSCs resulted in an accumulation of undifferentiated spermatogonia, suggesting impaired entry into the differentiating pathway (Sada et al. 2009). Although the key role of this posttranscriptional regulatory process in SSCs has been relatively well defined, the extrinsic signals controlling Nanos2 expression have not yet been identified. It has, however, been proposed that the characterized Nanos2 system is a relatively stable, cell autonomous mechanisms that is not effected by extrinsic spermatogenic signals regulating cycling between self-renewal and differentiation (Zhou et al. 2015a).

5.7 Implications of SSC Research from a Clinical Perspective

In understanding the biology and regulation of SSCs, it may be possible to harness these cells for use in therapeutic treatments; specifically for pre-pubertal boys with a cancer diagnosis that are subsequently facing chemotherapeutic intervention that may render them permanently infertile. While adult males facing such treatments have the opportunity to cryopreserve their spermatozoa for future use in IVF or ICSI, pre-pubertal boys are not yet producing sperm, thus do not have this option. As such, cryopreservation of tissue biopsies taken from the testes is a procedure that has begun to be offered to these patients in selected clinics worldwide (Ginsberg et al. 2010; Picton et al. 2015); despite the fact that treatments associated with such procedures remain purely experimental (Gassei and Orwig 2016). Theoretically, SSCs contained within testis tissue biopsies could be retrieved post-cryopreservation (many years in the future, when the patient has reached adulthood), proliferated in vitro, and injected back into the testis of the patient in order to recolonize the stem cell niche and thus reestablish fertility. Concerns surrounding this technique include the unknown capacity for human SSCs to remain viable within frozen tissue samples for long periods of time, the very low number of SSCs that are likely to be contained within a single tissue biopsy, the lack of characterized SSC-specific markers for human testis which could be used to isolate these cells specifically, the currently poor proliferation of putative human SSCs in culture, and the potential for reintroduction of cancer-causing cells into the patient when injecting spermatogonia back into the testes [reviewed by Sadri-Ardekani and Atala 2014]. A theoretical alternative to the autologous transplantation of cryopreserved spermatogonia is the initiation of in vitro spermatogenesis using these cells, followed by IVF or ICSI. However, while some recent success has been achieved with this technique in rodent models; producing haploid sperm that are fertile (Sato et al. 2011); concerns remain regarding the genetic integrity of these spermatozoa, particularly epigenetic changes that could have transgenerational effects [Reviewed by Cheung and Rennert 2011]. Clearly, with cancer survival rates for prepubertal patients now estimated to be above 80% (Howlander et al. 2016) due to ever-improving intervention strategies, a high importance is placed on the continuation of SSC research to fill the gaps-in-knowledge hindering therapeutic infertility treatments.

5.8 Conclusions

Regulation of SSC maintenance and self-renewal is not only integral for formation and expansion of the SSC pool during neonatal development, but is an absolute requirement for maintaining steady-state spermatogenesis in adulthood. The SSC pool, thought to be comprised by a subset of the A_{single} spermatogonial population, resides within the stem cell niche in the testis and responds to extrinsic cues; such as the growth factors Gdnf and Fgf2 to undergo self-renewal and sustain the reservoir from which the entirety of the spermatogenic lineage arises. We now have a comprehensive tool kit to study this rare subset of spermatogonia; using a suite of extrinsic and intrinsic markers to identify and isolate undifferentiated spermatogonial subsets, cell culture techniques to expand and sustain these cells, and transplantation and lineage tracing techniques to assess SSC content within heterogeneous spermatogonial populations. Perhaps the most useful platform for studying SSCs that has recently been developed is the formation of mouse lines with SSC-specific reporter transgenes; such as the Id4-eGfp mouse. For the first time, live populations of putatively pure SSCs can be isolated, and critically analyzed in comparison to progenitor populations and differentiating spermatogonial cell types. In the wake of these technologies we are likely to see an exponential increase in the identification of intrinsic regulatory molecules involved in the SSC fate decision. Improvement in our wealth of knowledge surrounding these processes will aid to facilitate advances in our current understanding of azoospermic infertility, and support the development of therapeutic strategies to treat this pathology, as well as provide options to pre-pubertal male chemotherapy recipients whose fertility may be compromised.

References

Aloisio GM, Nakada Y, Saatcioglu HD, Pena CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I, Hamra FK, Castrillon DH (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124(9):3929–3944

Amann RP (2008) The cycle of the seminiferous epithelium in humans: a need to revisit? J Androl 29(5):469–487

Aponte PM, Van Bragt MPA, De Rooij DG, Van Pelt AMM (2005) Spermatogonial stem cells: characteristics and experimental possibilities. APMIS 113(11–12):727–742

Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N, Hochedlinger K (2011) Sox2(+) adult stem/progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell 9(4):317–329

Ballow D, Meistrich ML, Matzuk M, Rajkovic A (2006) Sohlh1 is essential for spermatogonial differentiation. Dev Biol 294(1):161–167

Brinster RL (2002) Germline stem cell transplantation and transgenesis. Science 296(5576): 2174–2176

Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. PNAS 91(24):11303–11307

Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. PNAS 91(24):11298–11302

Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36(6):647–652

Chan F, Oatley MJ, Kaucher AV, Yang Q-E, Bieberich CJ, Shashikant CS, Oatley JM (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28(12):1351–1362

Chen L-Y, Willis WD, Eddy EM (2016) Targeting the Gdnf gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc Natl Acad Sci 113(7):1829–1834

Chen L, Brown PR, Willis WB, Eddy EM (2014) Peritubular Myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology 155(12):4964–4974

Chen, S., Liu, Y. (2015). Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction 149(4); R159–R167.

Cheung H-H, Rennert OM (2011) Generation of fertile sperm in a culture dish: clinical implications. Asian J Androl 13(4):618–619

Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126(5):669–680

Chiquoine AD (1954) The identification, origin, and migration of the primordial germ cells in the mouse embryo. Anat Rec 118(2):135–146

Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118(2):509–524

Clermont Y (1969) Two classes of spermatogonial stem cells in the monkey (Cercopithecus aethiops). Am J Anat 126(1):57–71

Clermont Y, Antar M (1973) Duration of the cycle of the seminiferous epithelium and the spermatogonial renewal in the monkey Macaca arctoides. Am J Anat 136(2):153–165

Cobrinik D (2005) Pocket proteins and cell cycle control. Oncogene 24(17):2796–2809

Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36(6):653–659

Crépieux P, Marion S, Martinat N, Fafeur V, Le Vern Y, Kerboeuf D, Guillou F, Reiter E (2001) The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. Oncogene 20(34):4696–4709

Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL, Porteus MH (2008) Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. Stem Cells 26(11):2928–2937

De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D (2004) Experimental approaches to the study of primordial germ cell lineage and proliferation. Hum Reprod Update 10(3):197–206

Dobrinski I, Ogawa T, Avarbock MR, Brinster RL (1999) Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. Mol Reprod Dev 53(2):142–148

Dorval-Coiffec I, Delcros J, Hakovirta H, Toppari J, Jégou B, Piquet-Pellorce C (2005) Identification of the leukemia inhibitory factor cell targets within the rat testis. Biol Reprod 72(3):602–611

Ebata KT, Zhang X, Nagano MC (2005) Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol Reprod Dev 72(2):171–181

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson EM Jr, Milbrandt J (1998) GFRα1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21(2):317–324

Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, Dolci S (2007) Repression of kit expression by Plzf in germ cells. Mol Cell Biol 27(19):6770–6781

Fuchs E (2009) The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137(5):811–819

Gassei K, Orwig KE (2013) SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PLoS One 8(1):e53976

Gassei K, Orwig KE (2016) Experimental methods to preserve male fertility and treat male factor infertility. Fertil Steril 105(2):256–266

Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, Brinster RL, Kolon TF (2010) An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod 25(1):37–41

Ginsburg M, Snow MH, McLaren A (1990) Primordial germ cells in the mouse embryo during gastrulation. Development 110(2):521–528
Glynn LE (1988) Natural history of the major histocompatibility complex. Jan Klein, John Wiley and Sons: New York. 775 pages, £90.75 (1986). Cell Biochem Funct 6(3):222–222
Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 121(9):3456–3466
Gonzalez-Herrera IG, Prado-Lourenco L, Pileur F, Conte C, Morin A, Cabon F, Prats H, Vagner S, Bayard F, Audigier S, Prats A (2006) Testosterone regulates FGF-2 expression during testis maturation by an IRES-dependent translational mechanism. FASEB J 20(3):476–478
Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, Vicini E (2012) Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction 143(3):325–332
Grisanti L, Falciatori I, Grasso M, Dovere L, Fera S, Muciaccia B, Fuso A, Berno V, Boitani C, Stefanini M, Vicini E (2009) Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27(12):3043–3052
Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M, Simons BD, Yoshida S (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14(5):658–672
He Z, Jiang J, Hofmann M, Dym M (2007) Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod 77(4):723–733
He Z, Jiang J, Kokkinaki M, Tang L, Zeng W, Gallicano I, Dobrinski I, Dym M (2013) MiRNA-20 and MiRNA-106a regulate spermatogonial stem cell renewal at the post-transcriptional level via targeting STAT3 and Ccnd1. Stem Cells 31(10). https://doi.org/10.1002/stem.1474
He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M (2010) Isolation, characterization, and culture of human spermatogonia. Biol Reprod 82(2):363–372
Helsel AR, Oatley MJ, Oatley JM (2017a) Glycolysis-Optimized Conditions Enhance Maintenance of Regenerative Integrity in Mouse Spermatogonial Stem Cells during Long-Term Culture. Stem Cell Reports 8(5):1430–1441
Helsel AR, Yang Q, Oatley MJ, Lord T, Sablitzky F, Oatley JM (2017b) ID4 levels dictate the stem to progenitor transition in the mammalian male germline. Development 144(4):624–634
Hermann BP, Sukhwani M, Simorangkir DR, Chu T, Plant TM, Orwig KE (2009) Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. Hum Reprod 24(7):1704–1716
Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G, Peterson K, Masterson K, Ramsey C, Ward T, Lienesch M, Volk A, Cooper DK, Thomson AW, Kiss JE, Penedo MCT, Schatten GP, Mitalipov S, Orwig KE (2012) Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11(5):715–726
Hobbs RM, Fagoonee S, Papa A, Webster K, Altruda F, Nishinakamura R, Chai L, Pandolfi PP (2012) Functional antagonism between Sall4 and Plzf defines germline progenitors. Cell Stem Cell 10(3):284–298
Hobbs RM, Seandel M, Falciatori I, Rafii S, Pandolfi PP (2010) Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell 142(3):468–479
Howlander N, Noone AM, Krapcho M, Miller D, Bishop K, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (2016) SEER cancer statistics review 1975–2013. N. C. Institute, Bethesda, MD
Hu Y-C, de Rooij DG, Page DC (2013) Tumor suppressor gene Rb is required for self-renewal of spermatogonial stem cells in mice. Proc Natl Acad Sci U S A 110(31):12685–12690
Huckins C (1971) The spermatogonial stem cell population in adult rats. Cell Prolif 4(4):335–349
Huszar JM, Payne CJ (2013) MicroRNA 146 (Mir146) modulates spermatogonial differentiation by retinoic acid in mice. Biol Reprod 88(1):15

Ishii K, Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2012) FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP 2K1 activation. Development 139(10):1734–1743

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41–49

Johnson L, Petty CS, Neaves WB (1980) A comparative study of daily sperm production and testicular composition in humans and rats. Biol Reprod 22(5):1233–1243

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T (2005) Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72(4):985–991

Kanatsu-Shinohara M, Mori Y, Shinohara T (2013) Enrichment of mouse spermatogonial stem cells based on aldehyde dehydrogenase activity. Biol Reprod 89(6):140, 141–110

Kanatsu-Shinohara M, Morimoto T, Toyokuni S, Shinohara T (2004b) Regulation of mouse spermatogonial stem cell self-renewing division by the pituitary gland. Biol Reprod 70(6):1731–1737

Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, Nakatsuji N, Horiuchi T, Shinohara T (2008b) Long-term culture of male germline stem cells from hamster testes. Biol Reprod 78(4):611–617

Kanatsu-Shinohara M, Takashima S, Ishii K, Shinohara T (2011) Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis. PLoS One 6(8):e23663

Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fässler R, Shinohara T (2008a) Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. Cell Stem Cell 3(5):533–542

Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004a) CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70(1):70–75

Kaucher AV, Oatley MJ, Oatley JM (2012) NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. Biol Reprod 86(5):164, 161–111

Kluin PM, de Rooij DG (1981) A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. Int J Androl 4(1–6):475–493

Kokkinaki M, Djourabtchi A, Golestaneh N (2011) Long-term culture of human SSEA-4 positive spermatogonial stem cells (SSCs). J Stem Cell Res Therapy 2(2):2488

Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. PNAS 100(11):6487–6492

Kubota H, Avarbock MR, Brinster RL (2004a) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731

Kubota H, Avarbock MR, Brinster RL (2004b) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. PNAS 101(47):16489–16494

Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL (2011) Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J 25(8):2604–2614

Li Y, Zhang Y, Zhang X, Sun J, Hao J (2014) BMP4/Smad signaling pathway induces the differentiation of mouse spermatogonial stem cells via Upregulation of Sohlh2. Anat Rec 297(4):749–757

Ling V, Neben S (1997) In vitro differentiation of embryonic stem cells: Immunophenotypic analysis of cultured embryoid bodies. J Cell Physiol 171(1):104–115

Loh Y, Wu Q, Chew J, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong K, Sung KW, Lee CWH, Zhao X, Chiu K, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei C, Ruan Y, Lim B, Ng H (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38(4):431–440

Lord T, Oatley JM (2017) A revised Asingle model to explain stem cell dynamics in the mouse male germline. Reproduction 154:R55–R64
Lovasco LA, Gustafson EA, Seymour KA, de Rooij DG, Freiman RN (2015) TAF4b is required for mouse spermatogonial stem cell development. Stem Cells 33(4):1267–1276
Lovelace DL, Gao Z, Mutoji K, Song YC, Ruan J, Hermann BP (2016) The regulatory repertoire of PLZF and SALL4 in undifferentiated spermatogonia. Development 143(11):1893–1906
Mäkelä J, Toppari J, Rivero-Müller A, Ventelä S (2014) Reconstruction of mouse testicular cellular microenvironments in long-term seminiferous tubule culture. PLoS One 9(3):e90088
Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MSH, Niwa H (2007) Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9(6):625–635
Matsui Y, Zsebo K, Hogan BLM (1992) Derivation of pluripotential embryonic stem-cells from murine primordial germ-cells in culture. Cell 70(5):841–847
Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493
Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382(6586):76–79
Murray P, Edgar D (2001) The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF). Differentiation 68(4–5):227–234
Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL (1998) Culture of mouse spermatogonial stem cells. Tissue Cell 30(4):389–397
Nagano MC (2003) Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69(2):701–707
Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S (2010) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328(5974):62–67
Nakatsuji N, Chuma S (2001) Differentiation of mouse primordial germ cells into female or male germ cells. Int J Dev Biol 45:541–548
Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95(3):379–391
Niedenberger BA, Busada JT, Geyer CB (2015) Marker expression reveals heterogeneity of spermatogonia in the neonatal mouse testis. Reproduction (Cambridge, England) 149(4):329–338
Niu Z, Goodyear SM, Rao S, Wu X, Tobias JW, Avarbock MR, Brinster RL (2011) MicroRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. Proc Natl Acad Sci 108(31):12740–12745
Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169:515–531
Oatley JM, Avarbock MR, Brinster RL (2007) Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on SRC family kinase signaling. J Biol Chem 282(35):25842–25851
Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL (2006) Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A 103(25):9524–9529
Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286
Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136(7):1191–1199
Oatley JM, Kaucher AV, Avarbock MR, Brinster RL (2010) Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. Biology of Reproduction 83(3):427–433
Oatley MJ, Racicot KE, Oatley JM (2011a) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84(4):639–645

Oatley MJ, Kaucher AV, Racicot KE, Oatley JM (2011b) Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. Biol Reprod 85(2):347–356

Ogawa T, Ohmura M, Yumura Y, Sawada H, Kubota Y (2003) Expansion of murine spermatogonial stem cells through serial transplantation. Biol Reprod 68(1):316–322

Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Schöler HR, Suda T (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice. Dev Biol 258(1):209–225

Paniagua R, Nistal M (1984) Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat 139(3):535–552

Panneerdoss S, Chang Y, Buddavarapu KC, Chen HH, Shetty G, Wang H, Chen Y, Kumar TR, Rao MK (2012) Androgen-responsive MicroRNAs in mouse sertoli cells. PLoS One 7(7):e41146

Paratcha G, Ledda F, Ibanez CF (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell 113(7):867–879

Pichel JG, Shen L, Sheng HZ, Granholm A-C, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382(6586):73–76

Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G, Rives N, Tournaye H, van Pelt AMM, Eichenlaub-Ritter U, Schlatt S (2015) A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod 30(11):2463–2475

Piquet-Pellorce C, Dorval-Coiffec I, Pham M, Jégou B (2000) Leukemia inhibitory factor expression and regulation within the testis. Endocrinology 141(3):1136–1141

Raverdeau M, Gely-Pernot A, Féret B, Dennefeld C, Benoit G, Davidson I, Chambon P, Mark M, Ghyselinck NB (2012) Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. PNAS 109(41):16582–16587

Raverot G, Weiss J, Park SY, Hurley L, Jameson JL (2005) Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 283(1):215–225

Ryu BY, Kubota H, Avarbock MR, Brinster RL (2005) Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. PNAS 102(40):14302–14307

Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24(6):1505–1511

Sada A, Suzuki A, Suzuki H, Saga Y (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325(5946):1394–1398

Sadri-Ardekani H, Atala A (2014) Testicular tissue cryopreservation and spermatogonial stem cell transplantation to restore fertility: from bench to bedside. Stem Cell Res Ther 5(3):68–68

Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. Nature 418(6895):293–300

Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, Matoba S, Ogura A, Ogawa T (2011) In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2:472

Schrans-Stassen BHGJ, van de Kant HJG, de Rooij DG, van Pelt AMM (1999) Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 140(12):5894–5900

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367(6461):380–383

Shinohara T, Avarbock MR, Brinster RL (1999) β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. PNAS 96(10):5504–5509

Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci 98(11):6186–6191

Skakkebæk N (1972) Possible carcinoma-in-situ of the testis. Lancet 300(7776):516–517

Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58–62

Sun F, Xu Q, Zhao D, Degui Chen C (2015) Id4 marks spermatogonial stem cells in the mouse testis. Nat Sci Rep 5(17594)

Suzuki H, Ahn HW, Chu T, Bowden W, Gassei K, Orwig KE, Rajkovic A (2012) SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. Dev Biol 361(2):301–312

Suzuki H, Sada A, Yoshida S, Saga Y (2009) The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Dev Biol 336(2):222–231

Tagelenbosch RAJ, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290(2):193–200

Takashima S, Hirose M, Ogonuki N, Ebisuya M, Inoue K, Kanatsu-Shinohara M, Tanaka T, Nishida E, Ogura A, Shinohara T (2013) Regulation of pluripotency in male germline stem cells by Dmrt1. Genes Dev 27(18):1949–1958

Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A, Shinohara T (2015) Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep 4(3):489–502

Tokuda M, Kadokawa Y, Kurahashi H, Marunouchi T (2007) CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76(1):130–141

Tong M, Yang Q, Davis JC, Griswold MD (2013) Retinol dehydrogenase 10 is indispensible for spermatogenesis in juvenile males. PNAS 110(2):543–548

Tseng Y-T, Liao H-F, Yu C-Y, Mo C-F, Lin S-P (2015) Epigenetic factors in the regulation of prospermatogonia and spermatogonial stem cells. Reproduction 150(3):R77–R91

Van Alphen MMA, Van De Kant HJG, De Rooij DG (1988) Follicle-stimulating hormone stimulates spermatogenesis in the adult monkey. Endocrinology 123(3):1449–1455

Vincent-Fabert C, Platet N, Vandevelde A, Poplineau M, Koubi M, Finetti P, Tiberi G, Imbert A-M, Bertucci F, Duprez E (2016) PLZF mutation alters mouse hematopoietic stem cell function and cell cycle progression. Blood 127(15):1881–1885

Wang L, Xu C (2015) Role of microRNAs in mammalian spermatogenesis and testicular germ cell tumors. Reproduction 149(3):R127–R137

Wang P, Suo L, Wang Y, Shang H, Li G, Hu J, Li Q (2014) Effects of GDNF and LIF on mouse spermatogonial stem cells proliferation in vitro. Cytotechnology 66(2):309–316

Wu Q, Song R, Ortogero N, Zheng H, Evanoff R, Small CL, Griswold MD, Namekawa SH, Royo H, Turner JM, Yan W (2012) The RNase III enzyme DROSHA is essential for MicroRNA production and spermatogenesis. J Biol Chem 287(30):25173–25190

Wu X, Goodyear SM, Tobias JW, Avarbock MR, Brinster RL (2011) Spermatogonial stem cell self-renewal requires ETV5-mediated downstream activation of Brachyury in mice. Biol Reprod 85(6):1114–1123

Wu X, Oatley JM, Oatley MJ, Kaucher AV, Avarbock MR, Brinster RL (2010) The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. Biol Reprod 82(6):1103–1111

Yamaji M, Tanaka T, Shigeta M, Chuma S, Saga Y, Saitou M (2010) Functional reconstruction of NANOS3 expression in the germ cell lineage by a novel transgenic reporter reveals distinct subcellular localizations of NANOS3. Reproduction 139(2):381–393

Yang Q-E, Gwost I, Oatley MJ, Oatley JM (2013b) Retinoblastoma protein (RB1) controls fate determination in stem cells and progenitors of the mouse male germline. Biol Reprod 89(5):113, 111–111

Yang Q, Racicot KE, Kaucher AV, Oatley MJ, Oatley JM (2013a) MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. Development 140(2):280–290

Yoshida S, Sukeno M, Nabeshima Y (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317:1722–1726

Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T, Nabeshima Y-i (2006) The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 133(8):1495–1505

Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T, Nabeshima Y (2004) Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 269(2):447–458

Yoshimizu T, Obinata M, Matsui Y (2001) Stage-specific tissue and cell interactions play key roles in mouse germ cell specification. Development 128(4):481–490

Youn H, Kim S-H, Choi KA, Kim S (2013) Characterization of Oct4-GFP spermatogonial stem cell line and its application in the reprogramming studies. J Cell Biochem 114(4):920–928

Yu M, Wang J, Liu W, Qin J, Zhou Q, Wang Y, Huang H, Chen W, Ma C (2014) Effects of tamoxifen on the sex determination gene and the activation of sex reversal in the developing gonad of mice. Toxicology 321:89–95

Zebedee Z, Hara E (2001) Id proteins in cell cycle control and cellular senescence. Oncogene 20(58):8317–8325

Zhang J, Tam W, Tong G, Wu Q, Chan H, Soh B, Lou Y, Yang J, Ma Y, Chai L, Ng H, Lufkin T, Robson P, Lim B (2006) Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nat Cell Biol 8(10):1114–1123

Zhang T, Oatley J, Bardwell VJ, Zarkower D (2016) DMRT1 is required for mouse spermatogonial stem cell maintenance and replenishment. PLoS Genet 12(9):e1006293

Zheng K, Wu X, Kaestner KH, Wang P (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9(1):1–11

Zheng Y, Thomas A, Schmidt CM, Dann CT (2014) Quantitative detection of human spermatogonia for optimization of spermatogonial stem cell culture. Hum Reprod 29(11):2497–2511

Zhou W, Shao H, Zhang D, Dong J, Cheng W, Wang L, Teng Y, Yu Z (2015b) PTEN signaling is required for the maintenance of spermatogonial stem cells in mouse, by regulating the expressions of PLZF and UTF1. Cell Biosci 5:42

Zhou Z, Shirakawa T, Ohbo K, Sada A, Wu Q, Hasegawa K, Saba R, Saga Y (2015a) RNA binding protein Nanos2 organizes post-transcriptional buffering system to retain primitive state of mouse spermatogonial stem cells. Dev Cell 34(1):96–107