REVIEW

Maintaining the male germline: regulation of spermatogonial stem cells

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Abstract

Spermatogonial stem cells (SSCs) are a self-renewing population of adult stem cells capable of producing progeny cells for sperm production throughout the life of the male. Regulation of the SSC population includes establishment and maintenance of a niche microenvironment in the seminiferous tubules of the testis. Signaling from somatic cells within the niche determines the fate of SSCs by either supporting self-renewal or initiating differentiation leading to meiotic entry and production of spermatozoa. Despite the importance of these processes, little is known about the biochemical and cellular mechanisms that govern SSC fate and identity. This review discusses research findings regarding systemic, endocrine, and local cues that stimulate somatic niche cells to produce factors that contribute to the homeostasis of SSCs in mammals. In addition to their importance for male fertility, SSCs represent a model for the investigation of adult stem cells because they can be maintained in culture, and the presence, proliferation, or loss of SSCs in a cell population can be determined with the use of a transplantation assay. Defining the mechanisms that regulate the self-renewal and differentiation of SSCs will fundamentally improve the understanding of male fertility and provide information about the regulation of adult stem cells in other tissues.

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Introduction

In animals, tissues require a continual supply of differentiated cells for function. The mammalian testis has a high rate of cell turnover and differentiation as spermatogonia differentiate into spermatozoa throughout the life of the male. The kinetics of sperm production were first described in rodents (Oakberg 1956) with the knowledge of the presence of adult stem cells, such as hematopoietic stem cells (HSCs), scientists hypothesized that germ cell differentiation in the testis required a stem cell population. The presence of a stem cell population responsible for continual sperm production in the testis was demonstrated in 1994 when Brinster & Zimmermann (1994) documented the first successful spermatogonial stem cell (SSC) transplantation in mice resulting in donor-derived spermatogenesis. Transplantation of a mixed population of germ cells that contain SSCs into the testis of a sterile mouse will restore fertility, although the genetics of the offspring will be of the donor (Avarbock *et al.* 1996).

Donor-derived spermatogenesis following transplantation is the true test to determine whether a *bona fide* SSC is present in a cell population. As with other stem cell populations, the ability of an SSC to colonize and initiate the formation of progeny cells depends on the microenvironment surrounding the SSC. The microenvironment, or niche, in the seminiferous tubule where SSCs are present appears to be critical for the production of progeny spermatogonia based on the difficulty of recapitulating this process *in vitro* or across species. However, the potential of SSCs does not appear to be limited to germ cells. Several research groups have reported that cells can be isolated from the testis and induced in culture to form embryonic-like stem cells with the ability to differentiate into multiple differentiated cell lineages (Kanatsu-Shinohara *et al.* 2004, Guan *et al.* 2006, Seandel *et al.* 2007). Indeed, transdifferentiation of stem/progenitor spermatogonia can produce tissues of all three germ layers, including prostatic, uterine, and skin epithelium as demonstrated in mice (Simon *et al.* 2009). These recent advances in the field underscore the potential use of SSCs for biomedical applications in regenerative medicine and provide a model to improve our basic understanding of adult stem cell populations.

Restoration of fertility following SSC transplantation in rodents suggests therapeutic potential for the technique in humans. Therapeutic uses of other adult stem cells such as HSCs motivate research to determine the basic mechanisms regulating the stem cells and the cells and factors that contribute to the stem cell niche (Yan & Owens 2008, Zon 2008). Similarly, identification of the downstream cellular signaling in SSCs following treatment with regulatory factors

such as glial cell line-derived neurotropic factor (GDNF) is being intensely studied because it will impact the ability to maintain human SSCs *in vitro* for therapeutic purposes (Orwig & Schlatt 2005, Kubota & Brinster 2006). Likewise, investigation of factors regulating SSCs in species besides mice suggests that comparative similarities will accelerate the application of SSC in both human health and agricultural species (Oatley *et al*. 2004, Ryu *et al*. 2005, Kanatsu-Shinohara *et al*. 2008).

This review will focus on SSC biology primarily depending on information gained from rodent models. We will emphasize several topics in the field where research findings have stimulated new hypotheses about the formation and maintenance of the SSC population. The importance of SSC function will be linked to the general understanding of spermatogenesis and the endocrine regulation of this process. For example, the formation of the SSC population and associated niche in the testis is critical to the initiation of spermatogenesis and the long-term production of sperm. Likewise, maintenance of the SSC population is likely regulated by different processes than the formation of the SSC population but equally important for the production of sperm throughout the life of the male. Also of particular emphasis in this review is the GDNF-regulated signaling involved in SSC proliferation and self-renewal because of its importance for SSC maintenance.

Spermatogonia

The continual production of sperm by sexually mature males requires an efficient and highly regulated process in the seminiferous tubules of the testis (Sharpe 1994). The somatic Sertoli cells of the seminiferous epithelium support the differentiation of germ cells from diploid undifferentiated spermatogonia to mature, haploid spermatozoa that are morphologically distinct from all other cells in the testis. In most species, this is a remarkably productive process high-lighted by species which produce over a billion sperm each day (Sharpe 1994). Thus, a constant supply of undifferentiated spermatogonia must be maintained so that these cells can differentiate into sperm (de Rooij & Russell 2000). The cell responsible for supplying undifferentiated spermatogonia is the SSC. After the prepubertal initiation of germ cell differentiation, spermatogenesis is maintained by the ability of SSCs to provide a continual supply of differentiating spermatogonia. This is a hallmark of a population of stem cells taken from an adult organ – the ability to endlessly self-renew to create additional stem cells and cells destined for differentiation (Fuchs *et al*. 2004). To maintain this ability, adult stem cells need to reside in a niche environment that supplies factors and provides interactions crucial for their survival and development. The niche is defined as the microenvironment that maintains the fate of the stem cell by preventing its differentiation and is usually composed of adjacent differentiated cells, the stem cells themselves, and

the extracellular matrix surrounding these cells. Niche environments vary depending on which organ or tissue the stem cell resides, and these niches are not always clearly defined (Spradling *et al*. 2001). Extrinsic regulation of the SSC self-renewal/differentiation process is thought to occur by the production of factors by somatic cells that contribute to the SSC niche in the seminiferous tubule (Shinohara *et al*. 2001, Spradling *et al*. 2001).

In the seminiferous tubule of the testis, germ cells are present at different phases of differentiation – from round, diploid spermatogonia near the basement membrane to haploid spermatozoa with a fully formed tail near the lumen. The SSC population is part of a subset of male germ cells called undifferentiated spermatogonia (de Rooij & Russell 2000). This subset includes A_{single} (A_s) spermatogonia that are thought to be the SSCs and their progeny cells A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia. The ultimate test for an adult stem cell is the ability to regenerate functional differentiated cells in the tissue of origin. Based on our current knowledge, SSCs (i.e. A_s spermatogonia) are capable of colonizing the seminiferous tubules in the testes of recipient mice resulting in long-term sperm production. In contrast, the conventional wisdom is A_{pr} or A_{al} spermatogonia are not capable of colonizing seminiferous tubules or subsequently producing sperm. Under normal conditions in the testis of an adult, the A_{al} spermatogonia differentiate without undergoing mitosis into type A_1 spermatogonia, the first spermatogonial cell type that is considered to be differentiated and associated with a stage of spermatogenesis (de Rooij & Grootegoed 1998). The differentiation pathway of undifferentiated spermatogonia and differentiated sperma-togonia provides the foundation of how thousands of sperm are produced from a single SSC. Morphological character-ization of the seminiferous tubules was used to estimate the number of A_s cells at $\sim 35\,000$ in the testis of a mouse (Meistrich & van Beek 1993) and $\sim 330\,000$ total type A_s, A_{pr}, A_{al} undifferentiated spermatogonia (Tegelenbosch & de Rooij 1993). Interestingly, although morphologically distinct from one another, there are numerous examples of proteins that are expressed across the undifferentiated spermatogonia lineage but not expressed in more differ-entiated spermatogonia (types A_1–A_4, Intermediate, B), spermatocytes, and spermatids (Table 1). These proteins therefore may serve as markers for an undifferentiated state but not a stem cell state.

Spermatogonial stem cells

As is evident from the descriptive name, A_{pr} spermatogonia are two undifferentiated spermatogonia derived from a single SSC that maintain a cytoplasmic bridge. The SSC would therefore also have the potential to divide and become two new SSCs. Based on the formation of A_{pr} spermatogonia from an SSC, it appears unlikely that a SSC can divide and form one A_{pr} spermatogonia and a new SSC. Thus, mammalian

Table 1 Genes associated with spermatogonial stem cells and undifferentiated spermatogonia. No gene product has been identified as spermatogonial stem cell specific; all genes in this list are expressed in the pool of undifferentiated spermatogonia

Gene symbol	Primary references
Zbtb16 (Plzf)	Buaas et al. (2004)
Neurog3 (Ngn3)	Yoshida et al. (2004)
Gfra1 (Gfrα1)	Naughton et al. (2006)
Ret	Naughton et al. (2006)
Utp14b (jsd)	Boettger-Tong et al. (2000) and Shetty et al. (2006b)
Pou5f1 (oct4)	Pesce et al. (1998)
Bcl6b	Oatley et al. (2006)
Etv5 (erm)	Oatley et al. (2007) and Schlesser et al. (2008)
Lhx1	Oatley et al. (2007)
Nanos2	Sada et al. (2009)

SSCs appear limited to symmetrical division, in which the parent stem cell forms either two new stem cells or two daughter cells committed to differentiation. However, we cannot be completely confident that mammalian SSC division occurs in this manner. For example, the male germline stem cell division in *Drosophila melanogaster* divides in an asymmetrical manner. In this system, the male germline stem cell is attached to a hub cell, and when signaled, it divides to become another germline stem cell or differentiates into a sperm cell (Tegelenbosch & de Rooij 1993, Fuller & Spradling 2007). Detailed illustrations of the potential symmetrical and asymmetrical division and the relationship of SSCs with other germ cells and somatic cells in the testis have been described (Oatley & Brinster 2008). The process of SSC self-renewal and commitment to differentiation is an important aspect of germ cell differentiation and the basis of two fundamental questions. First, what is the signal that stimulates an SSC to begin the process of differentiation? And second, what is the signal that stimulates an SSC to divide to self-renew the SSC population? In other words, how is an SSC controlled to divide into two A_{pr} spermatogonia or form two new SSCs? Thus, based on this description, once spermatogonia initiate the transition from a SSC to an A_{pr} spermatogonia, the daughter A_{pr} spermatogonia no longer have stem cell potential. This hypothesis is generally accepted; however, this is based on little or no experimental evidence.

A_s spermatogonia are considered the true SSC population, but it is unclear if all A_s spermatogonia are SSCs, or if a subpopulation of A_s spermatogonia have stem cell activity. To investigate the ability of undifferentiated spermatogonia to contribute to germline stem cell activity in the mouse, Nakagawa et al. (2007) used a transgenic mouse that expressed green fluorescent protein (GFP) under the regulation of the *Neurog3* (*Ngn3*) promoter after treating the mouse with tamoxifen. *Ngn3* is expressed by undifferentiated spermatogonia so this transgenic mouse model is useful for investigating the fate of undifferentiated spermatogonia by means of tracking GFP expression (Yoshida et al. 2004). Results of experiments in which the fate of germ cells following the induction of GFP that were injected into testes of recipient mice guides these researchers to conclude that there are two populations of SSCs in the mouse testis (Nakagawa et al. 2007). The first is an actual (or stable) SSC population, which is present throughout the life of the animal, and the ability of these cells to self-renew decreases as the animal ages. The second is a population of SSCs called potential germline stem cells, which is capable of self-renewal, but this activity does not occur under normal conditions. Instead, the second population of cells is characterized by a rapid turnover rate suggesting that they belong to a transit-amplifying, (rather than the dormant) population to provide differentiated spermatogonia. In contrast, potential germline stem cells shift their activity from differentiation to that of self-renewal (as stem cells) when the testis is damaged or if the cells are transplanted to another testis. In addition, the potential germline stem cells are responsible for supplying self-renewing stem cells to the testis as the actual SSCs are lost over time during normal aging (Nakagawa et al. 2007). Nakagawa et al. (2007) hypothesized that potential germline stem cells are found in the A_{pr} population. This hypothesis implies that the A_{pr} spermatogonia can break cytoplasmic bridges to gain the ability to self-renew. The enzymatic digestion of the testis used in SSC transplantation experiments breaks the cytoplasmic bridges between A_{pr} spermatogonia and all germ cells, which potentially stimulates A_{pr} spermatogonia to initiate self-renewal. Likewise, damage to the testis by chemical or other insult would provide a signal for A_{pr} spermatogonia to break bridges and initiate self-renewal. Therefore, as actual SSCs are lost due to aging, A_{pr} spermatogonia self-renewal could be stimulated by a variety of intrinsic or extrinsic cues including SSC death, differentiation or by systemic factors.

In addition to investigating the SSC populations in mice, Nakagawa et al. (2007) also used this system to estimate the number of cells in the actual SSC population. They concluded that the number of actual SSCs is ∼2000 cells, a number much lower than the 35 000 estimated for A_s spermatogonia based on morphological analysis of the seminiferous tubule (see above). The estimate by Nakagawa et al. (2007) is closer to another independently derived estimate of ∼3000 germ cells capable of colonizing the seminiferous testis of a recipient mouse (Nagano 2003). These data were obtained with the use of SSC transplantation, which based on interpretation of the findings by Nakagawa et al. (2007) may not accurately represent the actual or stable SSC population. Other factors also influence interpretation of SSC transplantation data, including SSC homing efficiency or the ability of a SSC to establish a niche in the testis of the recipient animal. Homing efficiency of SSCs in the seminiferous tubules of a recipient mouse has been estimated to be 10–12% and may vary depending on the age of the recipient or the technique used to eliminate the differentiating

germ cells from the testis of the recipient (Nagano 2003). For example, bulsulfan treatment or irradiation can eliminate endogenous spermatogenesis (Creemers *et al.* 2002, McLean 2008) but irradiation damages Sertoli cells reducing the ability of transplanted SSCs to colonize and initiate differentiation (Zhang *et al.* 2007). Comparison of SSC colonization between irradiated and bulsulfan-treated recipients requires consideration of somatic cell damage that alters SSC colonization efficiency and subsequent differentiation. More-over, the true number of SSCs, actual or potential, can only be determined if an SSC-specific marker is identified.

GDNF-regulated SSC self-renewal

The second fundamental question about SSC self-renewal – what is the signal that stimulates an SSC to divide and replenish the SSC population, has been at least partially answered with the use of *in vivo* mouse models and work investigating SSC self-renewal *in vitro*. As will be described in more detail below, GDNF is an essential factor for the proliferation and simultaneous self-renewal of SSCs. It is difficult to consider the question of SSC differentiation and SSC self-renewal independently. For example, it is not known if the loss of direct GDNF signaling *in vivo* to a SSC is the primary determinant leading to the differentiation of SSCs or if other factors are involved. It appears that when SSCs are cultured under serum-free conditions in a defined medium containing GDNF, soluble GFRα1, and basic fibroblast growth factor (bFGF) that the removal of GDNF from the culture leads to changes in the appearance of SSCs such that they no longer maintain cell contacts or clumps of cells (Oatley *et al.* 2006). This result was verified independently using slightly different culture conditions for SSCs that included GDNF, epidermal growth factor, and bFGF, in which removal of GDNF resulted in a significantly decreased number of SSCs *in vitro* (Lee *et al.* 2007). The situation *in vivo* is likely more complex because it would be difficult to envision a mechanism that would completely remove GDNF from the SSC niche microenvironment. However, GDNF may be sequestered in the niche by the extracellular matrix creating a gradient that results in SSC differentiation into spermatogonia as cells move away from high concentrations of GDNF. Such a mechanism may be necessary because the expression of GDNF and its cognate receptors GFRα1 and RET does not appear to be stage specific or associated within limited regions of the seminifer-ous tubule (Meng *et al.* 2000, Fouchecourt *et al.* 2006, Naughton *et al.* 2006). Although there are differences in the expression of these proteins between species, the expression patterns of GDNF, GFRα1, and RET in somatic and germ cells of the testis suggest that other factors are involved in signaling SSCs to commit to the spermatogonial differentiation pathway (Fig. 1). Alternatively, it is important to note that the expression patterns of RET detected by a general anti–RET antibody and a phosphorylation-specific RET antibody are different. The phosphorylation-specific anti-RET staining
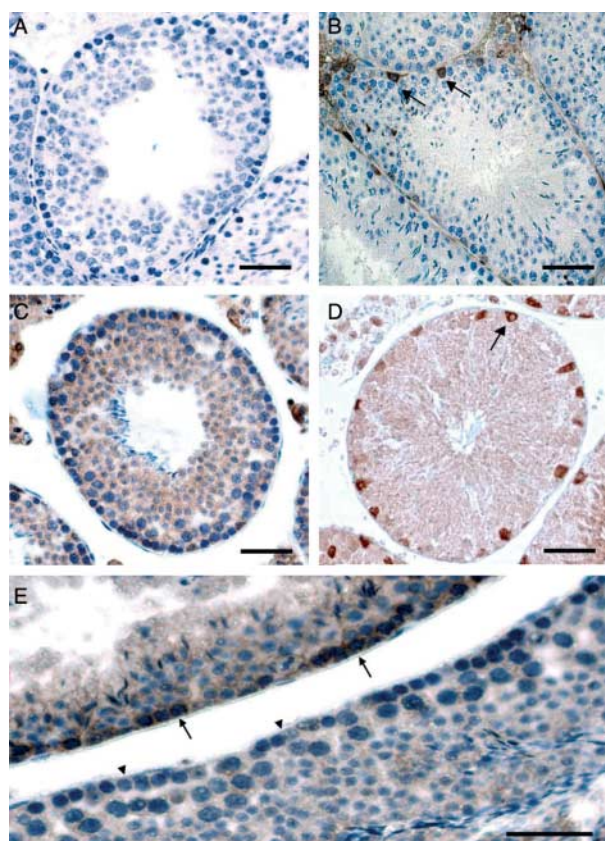

Figure 1 Photomicrographs of cross-sections of seminiferous tubules in an adult mouse testis used for immunohistochemical analysis. (A) Control hematoxylin counter-stained mouse testis section with no primary antibody incubation. (B) Immunohistor-chemistry staining for PLZF. Undifferentiated spermatogonia positive for PLZF (arrows) are found along the basement membrane preferentially localized to the interstitial space. The image also demonstrates that not all seminiferous tubules contain PLZF-positive spermatogonia. Cells with positive stain in the interstitial space are non-specific, while staining in seminiferous tubules is specific for PLZF based on controls. (C) Immunohistochemistry staining for GDNF. Somatic and germ cells are positive for GDNF in all seminiferous tubules regardless of stage. (D) Immunohisto-chemistry for RET. Spermatogonia along the basement membrane are positive for RET (arrows) with some expression visualized in Sertoli cells. No counterstain was used to assist in the visualization of RET-positive cells. (E) Immunohistochemistry staining for phosphorylation-specific Tyrosine 1062 (Y-1062) residue of RET in germ and somatic cells. The importance of intracellular signaling via Y-1062 in RET has been demonstrated for spermatogonial stem cell self-renewal in mammals. The image illustrates that expression of RET phosphorylated in Y-1062 is heterogeneous among seminiferous tubules. Spermatogonia positive for RET-Y-1062 staining are indicated with an arrow, and negative spermatogonia in the adjacent tubule are indicated with an arrowhead. Scale bar is 50 μm in all panels. Images are representative of independent experiments ($n=3$).

has stage-specific characteristics in contrast to the staining observed with the use of the general anti–RET antibody (Fig. 1). This raises the possibility that the phosphorylation and subsequent dephosphorylation of RET may be important

cellular mechanisms to regulate the self-renewal or differentiation of SSCs in a niche- or stage-specific way.

GDNF expression in the testis needs to be tightly regulated for normal germ cell differentiation and sperm production. An accumulation of undifferentiated spermatogonia due to the inability to differentiate occurs in mice genetically modified to overexpress GDNF resulting in infertility (Meng *et al*. 2000). Interestingly, attempts to induce spermatogonial differentiation with retinoic acid (RA) treatment in the GDNF overexpressing mice resulted in apoptosis of undifferentiated spermatogonial clumps (Meng *et al*. 2000). RA stimulates undifferentiated spermatogonia to differentiate into type A_1 spermatogonia, the first differentiating germ cell that is associated with stages of the seminiferous epithelium. Therefore, GDNF and RA signaling represents two independent means for regulating germ cell maturation, and abnormal expression of GDNF suppresses spermatogonial differentiation.

Transplantation of SSCs from transgenic mice overexpressing GDNF described by Meng *et al*. (2000) into the testes of irradiated mice resulted in successful colonization of the donor cells. However, undifferentiated spermatogonia formed clumps in the seminiferous tubules of recipient testes similar to the phenotype observed in the transgenic donor mice (Creemers *et al*. 2002). These results suggest that high expression of GDNF in spermatogonia is the basis of the phenotype observed in this transgenic mouse line. Both Sertoli cells and differentiating germ cells express GDNF mRNA and protein in the rat indicating that paracrine and autocrine regulation occurs in these cell types (Fouchecourt *et al*. 2006). Precise SSC transplantation experiments using cell-sorting approaches to enrich for SSCs could be used to determine if the proportion of SSCs in the GDNF over-expressing mice was higher than in control mice of similar age. This experimental approach would demonstrate if the SSC population *in vivo* could be manipulated by altering the concentration of factors known to regulate SSC activity within the niche. The GDNF overexpression transgenic mouse model demonstrates that the precise expression of GDNF is important for spermatogonial differentiation and normal germ cell maturation, especially considering that as the transgenic mice age, they develop testicular tumors (Meng *et al*. 2001).

GDNF signaling in SSCs

Based on the importance of GDNF for SSC proliferation/self-renewal and to maintain SSCs in culture, the cell signaling cascade activated by this factor is of intense interest. In spermatogonia, GDNF binds to GFRα1, which is linked to the plasma membrane by a glycosyl-phosphatidylinositol anchor (Airaksinen & Saarma 2002). The GDNF–GFRα1 complex recruits two molecules of the receptor tyrosine kinase RET to lipid rafts in the plasma membrane, thereby activating autophosphorylation and intracellular signaling. Cellular and physiological actions of RET activation by GDNF binding have been demonstrated in the kidney, neural

tissue, and the testis (Sariola & Saarma 2003). RET is a transmembrane receptor tyrosine kinase that is subject to alternative splicing resulting in two forms of the protein, RET9 and RET51 (Santoro *et al*. 2004), and both forms of RET are expressed in SSCs (Oatley *et al*. 2007). RET has a typical intracellular kinase domain with 12 autophosphorylation sites that can activate multiple signaling pathways (Santoro *et al*. 2004). Mutations of the *ret* gene can lead to cancer in multiple tissues including thyroid, intestine, and neurons associated with other tissues (Santoro *et al*. 2004). Specific alterations to the RET protein also alter germ cell differentiation. For example, mice heterozygous for mutations in two amino acid residues within the cytoplasmic tail of RET had fewer spermatogonia at day 10 after birth when compared to controls (Jain *et al*. 2004). Further verification of the importance of GDNF, GFRα1 and RET for spermatogonial proliferation and survival was demonstrated with the use of whole testis transplantation of testes from GDNF−/−, GFRα1−/−, and RET−/− mice at the day of birth. These experiments demonstrated that during the first week of testis development, there is significant germ cell depletion due to the lack of spermatogonial proliferation. However, it was not confirmed by SSC transplantation if germ cell depletion was due to the inability of SSCs to maintain undifferentiated state or loss of SSCs (Naughton *et al*. 2006).

Downstream signaling pathways potentially activated by RET tyrosine phosphorylation include Janus kinase/signal transducer and activator of transcription, Src family kinase (SFK), mitogen-activated protein kinase (MAPK), protein kinase C, and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt; reviewed in Ichihara *et al*. (2004) and Arighi *et al*. (2005)). Investigations of the downstream signaling in SSCs and undifferentiated spermatogonia following GDNF treatment have demonstrated that at least two of the signaling pathways are active in SSCs and undifferentiated spermatogonia. However, the biological outcome of the activation of different signaling pathways in SSCs by GDNF indicates that in addition to SSC self-renewal, GDNF is also important for basic SSC survival and function (Oatley *et al*. 2007).

Microarray gene expression profiling was used to identify genes regulated by GDNF that are potentially involved in SSC proliferation and survival (Oatley *et al*. 2007). These researchers established SSC cultures with the use of a serum-free medium containing GDNF, GFRα1, and bFGF and then eliminated GDNF signaling in these cells by removing GDNF and soluble GFRα1 from the medium for 18 h. As expected, the expression of over 270 genes was significantly changed by the loss of GDNF-induced signaling. To identify genes induced by GDNF, gene expression profiles were obtained for SSC cultures 2, 4, and 8 h after GDNF and GFRα1 were added back to the culture medium. A group of genes with patterns of expression distinctly regulated by GDNF stimulation were identified (Oatley *et al*. 2007). Functional characterization of one of these genes, *Bcl6b*, with the use of RNA interference and SSC transplantation demonstrated that suppressing *Bcl6b* expression results in a

loss of SSCs in culture (Oatley *et al*. 2007). In addition, targeted deletion of *Bcl6b* in mice results in smaller testes and seminiferous tubules with impaired germ cell differentiation. These results support the hypothesis that *Bcl6b* is important for SSC function; however, since the knockout mice are fertile, *Bcl6b* is likely not necessary for SSC self-renewal and proliferation but important for maximum sperm production.

As discussed above, multiple signaling cascades can be induced by GDNF via its interaction with RET. Investigation of downstream signaling in SSCs following GDNF treatment has yielded interestingly contradictory results with regard to cell self-renewal and proliferation versus survival. These differences may be due to purity of the undifferentiated spermatogonia in culture or the strain of mouse used by respective research groups. SSCs obtained from DBA/2 mice can survive in culture for long periods with or without fibroblast feeder layers (Kanatsu-Shinohara *et al*. 2005). Removal of GDNF from these cultures significantly reduced SSC proliferation; however, inhibition of MEK signaling in the presence of GDNF with the specific inhibitor PD098059 did not alter SSC proliferation (Lee *et al*. 2007). Regulation of SSC proliferation in culture through the PI3K pathway was investigated with the PI3K inhibitor LY294002. Interestingly, LY294002 did inhibit SSC growth but did not stimulate differentiation of SSCs (Lee *et al*. 2007). Subsequent analysis demonstrated that GDNF treatment resulted in the phosphorylation of AKT, and that treating SSC cultures with LY294002 resulted in fewer colonies in recipient testes following SSC transplantation compared to controls. These data suggest that the activation of the PI3K pathway is important for SSC survival in culture (Lee *et al*. 2007). Oatley *et al*. (2007) also demonstrated that AKT is phosphorylated through the PI3K signaling cascade in SSCs following GDNF treatment. In addition, Oatley *et al*. (2007) showed that inhibition of AKT with a specific inhibitor significantly reduced the number of SSCs present in cultures as determined by the SSC transplantation assay. Assaying the expression of GDNF-induced genes in SSCs following treatment with the AKT inhibitor demonstrated that inhibition of AKT suppressed GDNF stimulation of *Bcl6b*, *Erm*, and *Lhx1* (Oatley *et al*. 2007). Interestingly, inhibition of AKT also suppressed the expression of two genes in SSCs, *Plzf* and *Pou5f1*, which are not regulated by GDNF. The authors demonstrated that *Bcl6b*, *Erm*, and *Lhx1* are important for SSC renewal, because suppressing their expression with the use of RNA interference resulted in fewer SSCs as determined by SSC transplantation. The result that inhibition of AKT resulted in the suppression of GDNF-regulated and non-GDNF-regulated genes in SSCs guided these researchers to conclude that AKT is important for basic SSC survival but not necessarily for SSC self-renewal.

GDNF can activate multiple signaling pathways through its interaction with RET. A signaling pathway investigated in SSCs following GDNF treatment involves the SFKs. SSCs cultured in the presence of GDNF express the SFK members *c-Src*, *Yes*, *Fyn*, *Lyn*, and *Hck* and GDNF treatment

of SSC cultures following overnight withdrawal of GDNF demonstrated that *c-Src* is phosphorylated after GDNF treatment (Oatley *et al*. 2007). Activation of SFKs is functionally significant in SSCs as demonstrated with treatment of SU6655, a selective chemical inhibitor of SFKs. Not only did SU6644 treatment block phosphorylation of AKT it also blocked GDNF induction of *Bcl6b*, *Erm*, and *Lhx1* and reduced the number of SSCs in culture (Oatley *et al*. 2007). In contrast to inhibition of AKT (see previous paragraph), inhibition of SFKs did not suppress expression of *Plzf* and *Pou5f1* in SSC cultures. These data support the hypothesis that SFKs are important for SSC self-renewal in addition to mediating the Akt-supported general survival of SSCs (Oatley *et al*. 2007). Src phosphorylation also appears to be a pre requisite for PI3K/AKT signaling and induction of N-myc expression in GDNF treated, GRFα1-positive spermatogomia isolated from the testis of mice 4·5 to 5 days after birth (Braydich-Stolle *et al*. 2007) Thus, activation of an Src/PI3K-dependent pathway involving AKT phosphorylation is critical to support GDNF-induced ssc proliferation (Braydich-Stolle *et al*. 2007).

RET is phosphorylated at multiple tyrosine residues on the cytoplasmic tail of the protein, and these phosphorylation sites serve as binding sites for proteins that regulate downstream signaling cascades (Ichihara*et al*. 2004). Mutation of RET tyrosine 1062 results in the loss of germ cells during the first 4 weeks of life such that there are no differentiating germ cells by day 28 after birth (Jijiwa *et al*. 2004). Interestingly, there was no increase in apoptosis in the seminiferous tubules of RET tyrosine 1062 mutant mice compared to controls suggesting that the SSCs in tyrosine 1062 mutant mice differentiate at an accelerated rate leading to depletion or exhaustion of the SSC population early in life (Jijiwa *et al*. 2004). Therefore, mutation of the RET tyrosine 1062 may not disrupt the formation of the SSC population in neonatal mice because spermatocytes are present in tyrosine 1062 mutant mice 14 days after birth. Likewise, due to the fact that RET is expressed in A_{pr} and A_{al} spermatogonia as well as SSCs, the observed phenotype may be independent of SSCs and represent the inability of undifferentiated spermatogonia to initiate the differentiation process to eventually become meiotic germ cells. GDNF signaling appears to regulate the survival and maintain germ cell fate in SSCs (Oatley *et al*. 2007), and tyrosine 1062 may be a key regulation point for both functions. Thus, further investigation of SSCs from the RET tyrosine 1062 mutant such as SSC transplantation experiments to determine if SSCs are present in post pubertal animals would be instrumental in determining if the primary GDNF signal transduction in SSCs occurs through tyrosine 1062.

SSC gene expression profiling

Gene expression profiling has also been investigated in GFRα1-selected spermatogonia (Hofmann *et al*. 2005). A combination of cell separation with a BSA gradient and

selection for GFRα1-positive cells with magnetic beads was used to isolate spermatogonia from mice 6 days after birth. These cells were incubated with GDNF for 10 h in medium containing 10% NuSerum, and the RNA from these cells was used to identify GDNF-regulated genes. As determined with the use of gene microarrays, over 1100 genes were differentially regulated by GDNF compared to controls (Hofmann *et al.* 2005).

These data are interesting in comparison with the gene microarray analysis of SSCs cultured with GDNF conducted by Oatley *et al.* (2006). Based on the comparison of published lists of regulated genes, the genes with the largest change in expression following GDNF treatment are not consistent between studies. This could be due to the different isolation and culture conditions in the two studies. Hofmann *et al.* (2005) treated freshly isolated GFRα1-positive spermatogonia with GDNF for 10 h, while Oatley *et al.* (2006) treated SSCs that had been cultured for several weeks. In addition, Oatley *et al.* (2006) analyzed gene expression in cultured SSCs at shorter intervals (2, 4, and 8 h) than Hofmann *et al.* (2005), suggesting that GDNF could induce the expression of a subset of early immediate genes and additional genes that are downstream of the initial group of genes. Oatley *et al.* (2006) used a serum-free culture that only included exogenous bFGF in addition to the GDNF and soluble GFRα1, while Hofmann *et al.* (2005) did not add exogenous bFGF or soluble GFRα1 but did supplement the medium with 10% NuSerum. Although different culture conditions can support SSCs, the inclusion of serum has been shown to reduce SSC proliferation or survival *in vitro* (Kubota *et al.* 2004).

Hofmann *et al.* (2005) reported that ∼50% of the GFRα1 cells were positive for kit, while Oatley *et al.* (2006) reported that the cultured SSCs in their study were negative for kit protein based on immunofluorescent staining. Two reports demonstrated that kit-positive cells from adult cryptorchid mice did not have SSC colonization activity potential based on SSC transplantation experiments (Shinohara *et al.* 2000, Kubota *et al.* 2003). A similar study demonstrated that the kit-positive cells from the testes of mice at 7·5 days of age did not colonize the testes of recipient mice (Ohbo *et al.* 2003). Thus, the cell population in the Hoffman *et al.* (2005) gene profiling experiments may have been a mixed germ cell population including differentiated spermatogonia and undifferentiated spermatogonia. Also, the GFRα1-positive cells were isolated from the testes of mice 6 days after birth when the hierarchy of the germ cell population has not been completely established. Indeed, the first wave of spermatogenesis is less efficient than subsequent rounds with several interesting differences (de Rooij & Russell 2000). Expression of some genes associated with spermatogonia differentiation in neonatal germ cells is different than germ cells from adults (Yoshida *et al.* 2006), and a wave of apoptosis occurs in germ cells during early testis development (Mori *et al.* 1997, Rodriguez *et al.* 1997, Russell *et al.* 2002). Therefore, it is possible, especially in different strains of mice, that during the first week of life, gene expression in SSCs and undifferentiated

spermatogonia may not be as tightly regulated as in adult mice. These differences may partially explain the variation in gene expression responses to GDNF reported in these two studies.

Crosstalk and GDNF-independent signaling in SSCs

The activation of several signaling cascades by GDNF binding to GFRα1 and RET raises the possibility that crosstalk between signaling pathways may regulate SSCs. For example, in the developing kidney, crosstalk between vascular endothelial growth factor (VEGF) and GDNF occurs in ureteric bud-derived cells (Tufro *et al.* 2007). VEGF induces phosphorylation at RET tyrosine 1062, and both binding of VEGF to its receptor KDR and binding of GDNF to RET have an additive effect on phosphorylation at tyrosine 1062. In rodents, Leydig and Sertoli cells produce VEGF, and Leydig expression is stimulated by human chorionic gonadotropin (hCG) treatment (Nalbandian *et al.* 2003, Rudolfsson *et al.* 2004). In mice and human testes, Leydig cells express the VEGF receptors KDR and FLT1 (Ergun *et al.* 1997, Korpelainen *et al.* 1998). We have shown that VEGF treatment of bovine testis tissue increases germ cell differentiation resulting in more sperm produced in testis tissue grafts (Schmidt *et al.* 2006). Based on these results and expression of KDR by spermatogonia, we hypothesized that VEGF stimulates spermatogonia proliferation. Treating testis tissue from 4- to 8-week calves that contain spermatogonia as the only germ cell with VEGF resulted in an increase in spermatogonia compared to controls treated with vehicle (Caires *et al.* 2009). VEGF induces the expression of genes that promote germ cell survival in the testis tissue (Caires *et al.* 2009), suggesting that one nonvascular role of VEGF signaling in the testis is promoting spermatogonial survival during testis development. Thus, the interaction between GDNF and VEGF may promote general cell survival in SSCs as well as undifferentiated spermatogonia.

GDNF signaling independent of RET in cells that express GFRα1 has been demonstrated *in vivo* and *in vitro* (Paratcha & Ledda 2008). Alternatively to RET binding, the GDNF/GFRa1 complex can bind with neural cell adhesion molecule (NCAM) to stimulate signaling in cultured Schwann cells and stimulate axonal in hippocampal and cortical neurons in addition to other biological functions (Paratcha *et al.* 2003, Paratcha & Ledda 2008). Cultured SSCs from mice and rats express NCAM suggesting that GDNF may mediate changes in SSCs through interaction with NCAM. GDNF binding to NCAM is stabilized by GFRα1, and this interaction causes a reduction in NCAM-regulated homophilic cell adhesion (Sjostrand *et al.* 2007). The interaction between NCAM and GDNF in the testis may regulate the migration of SSCs away from the SCC niche when cellular differentiation program initiates to switch SSC activity away from GDNF-regulated self-renewal. Alternatively, parallel signaling pathways could also be activated to regulate cell fate.

Regulation of GDNF expression

Cellular and physiological actions of RET activation by GDNF binding have been demonstrated in the kidney, neural tissue, and the testis (Sariola & Saarma 2003). While is it known that GDNF is very important in SSC biological activity in the testis, little is known about what regulates its expression in this tissue. GDNF mRNA and protein have been localized in Sertoli cells (Meng *et al.* 2000), spermatogonia, and spermatids in the mouse (Yu *et al.* 2003). Similar results have been reported for rats and humans (Fouchecourt *et al.* 2006). As shown in Fig. 1, the expression pattern of GDNF is not restricted to germ cells, while RET is expressed in a subset of spermatogonia. The expression of the phosphorylated form of RET varies between seminiferous tubules, suggesting that it has a stage-specific regulation (Fig. 1). This suggests that the regulation of SSC proliferation and self-renewal may be more dependent on the expression and phosphorylation of the receptors rather than the availability of GDNF in the seminiferous tubule.

FSH increases the expression of GDNF 3 h after treatment in cultures of primary Sertoli cells from 14-day-old mice (Tadokoro *et al.* 2002). In fact, these researchers suggest that FSH stimulation via homeostatic control is a major regulator of GDNF concentration in the testis. However, this conclusion is based on data in which a GNRH antagonist (Nal-Glu) is used to suppress FSH. This approach would also suppress testosterone production so the role of testosterone in this homeostatic regulation is not known (Tadokoro *et al.* 2002).

In another study, the mouse Sertoli cell line TM4 was used to investigate regulation of GDNF expression. FGF2 at 25 ng/ml stimulated GDNF expression fivefold in TM4 cells 48 h after treatment, while FSH stimulated GDNF expression twofold in TM4 cells 3–24 h after treatment (Simon *et al.* 2007). Stimulation of GDNF expression by FGF2 in TM4 cells was suppressed by pretreatment of the TM4 cells with the PI3K inhibitor wortmannin and the MAPK signaling inhibitor PD98059. However, neither inhibitor alone or when added together completely abolished the FGF2 stimulated increase of GDNF expression. This indicates that other cellular mechanisms in addition to the MAPK and PI3K pathways are involved in FGF2 induction of GDNF expression. FSH increased GDNF expression 2·1-fold 6 h after treatment in primary Sertoli cells from 10-day-old mice. Similarly, FGF2 treated resulted in a 1·92-fold increase in *Gdnf* mRNA levels 48 h after treatment (Simon *et al.* 2007). However, experiments with wortmannin and PD98059 were not performed with FGF2- or FSH-treated primary mouse Sertoli cells.

The role of FGF2 regulation of GDNF expression in the testis may be complex based on the fact that FGF2 has been shown to play a role in SSC survival *in vitro* (Kubota *et al.* 2004, Hofmann *et al.* 2005) but SSCs do not express GDNF (Oatley *et al.* 2006). Similarly, the role of FGF2 in the testis is complicated by the fact that the FGF2 knockout mouse is fertile (Ortega *et al.* 1998). Thus, information regarding the cellular and molecular mechanisms regulating GDNF expression in the testis is lacking and represents a gap in the field. Similarly, information about GDNF expression by Sertoli cells is based on experimentation using prepubertal rodents as the source of Sertoli cells. Sertoli cell response to FSH declines in sexually mature animals; however, it is still biologically important, especially for spermatogonial differentiation. Therefore, details about how FSH may contribute to maintenance of SSCs by regulating the expression of GDNF or other factors in sexually mature animals have not been determined.

Retinol and retinoic acid

Vitamin A is essential for male fertility and normal spermatogenesis in rats and mice (Morales & Griswold 1987, van Pelt & de Rooij 1990). RA, an active metabolite of vitamin A, is critical for the differentiation of spermatogonia and entry of germ cells into meiosis (Bowles & Koopman 2007). Vitamin A deficiency in rats results in a block of germ cell differentiation such that only spermatogonia and scant preleptotene spermatocytes are present while mice have only undifferentiated spermatogonia. RA receptors are expressed in both germ and somatic cells in the testis (Dufour & Kim 1999), so RA can regulate germ cells directly or regulate the somatic cells that contribute to the SSC niche. According to microarray data, cultured SSCs express RXRα, RXRβ, and RARα, and the expression of these genes is not regulated by GDNF (Oatley *et al.* 2006). Therefore, there is potential of direct RA regulation of SSCs. Dann *et al.* (2008) demonstrated that repressing *Pou5f1* with shRNA resulted in a reduction of the ability of cultured spermatogonia to colonize the testes of recipient mice after transplantation. In addition, RA represses *Pou5f1* expression in cultured spermatogonia supporting the hypothesis that RA stimulates differentiation of SSCs (Dann *et al.* 2008). These data suggest a second mechanism that may regulate SSC differentiation in addition to the loss of GDNF signaling especially considering the fact that the expression of *Pou5f1* did not change in SSCs when GDNF/GFRα1 were removed and added back to SSC cultures (Oatley *et al.* 2006). As with other publications described previously in this review, there are several differences between the culture conditions, analysis of transplantation data and the strain of mice used for spermatogonial enrichment. For example, the use of DBA/2 (Dann *et al.* 2008) versus C57Bl/6 (Oatley *et al.* 2006) mice as the source of SSCs complicates the comparison of the data and may provide explanation for contradictory data.

The block in spermatogonial differentiation in vitamin A-deficient mice provides a unique animal model to investigate RA regulation of SSCs *in vivo*. We hypothesized that the testis of vitamin A-deficient mice would be naturally enriched for SSCs based on the loss of differentiating germ

cells in this model. Similarly, the testes of neonatal mice and cryptorchid testes in adult mice are enriched for SSCs because differentiating germ cells are not present (Shinohara *et al.* 2000, McLean *et al.* 2003). Unexpectedly, as demonstrated by SSC transplantation, the number of biologically active SSCs in the testes of vitamin A-deficient mice was significantly less than that would be expected based on the regression of the testis (McLean *et al.* 2002). Based on the fact that some receptors for RA are expressed in SSCs and somatic cells that comprise the niche, it is not known if the lack of RA signaling in vitamin A-deficient mice directly to the SSCs or to the somatic cells is the cause of the loss of SSCs in this model. The vitamin A-deficient mice must be the offspring of breeding pairs maintained on vitamin A-deficient diet prior to fertilization, during gestation and through lactation. Approximately 12 weeks after weaning and maintenance on vitamin A-deficient diet, testicular regression is complete in the deficient animals. Close inspection of this protocol reveals that that vitamin A-deficient mice are not exposed to RA during the formation of the SSC population in neonatal mice or in the maintenance of the SSC population in adults. Thus, RA may be important for the formation of the SSC population that occurs during the first 3–4 days of life in mice (McLean *et al.* 2003) or in the maintenance of the SSC population in adults or both.

Vasculature–associated niche

The lack of a specific marker for SSCs and the small number of SSCs present in the testis have prevented identification of the physical location of the SSC niche in the seminiferous tubule. In other adult stem cell systems, the niche can be comprised of multiple somatic cells and extracellular matrix (ECM) components. The synthesis and turnover rate of growth factors and other biologically active molecules produced in the niche microenvironment could also be regulated by changes in local tissue activity and, or in response to extrinsic cues. The potential regulation of the SSC niche with systemic endocrine factors suggests that the physical location of SSCs and the niche may need to be in close proximity to the vascular system. This hypothesis was tested with the use of three-dimensional culture system and image capturing system (Yoshida *et al.* 2007). These researchers localized undifferentiated spermatogonia in transgenic mice expressing GFP under the control of the *Ngn3* regulatory sequence. Undifferentiated spermatogonia are preferentially localized to the vascular network and interstitial space surrounding the seminiferous tubules (Yoshida *et al.* 2007). Over time, they observed that differentiating spermatogonia move from the area of the seminiferous tubule associated with the vascular network into a random pattern throughout the tubule. We have seen a similar cell localization pattern for cells positive for the undifferentiated spermatogonial expressed protein PLZF in adult mice (Fig. 1). Approximately 20% of the tubules in a total testis cross-section (20 out of 125 total

tubules) have germ cells positive for PLZF, and on average, about one germ cell per tubule in a cross-section is PLZF positive. The majority of these germ cells are close to the interstitial space, although two-dimensional morphological images must be evaluated with caution due to the inability to view structures above and below the tissue section.

The close association of the niche to the vasculature and interstitial cells, including Leydig cells, hypothesized by Yoshida *et al.* (2007) implies that transfer of factors transported in the blood or from the interstitial cells is critical for SSC maintenance, differentiation, or both. This organization has been proposed for other adult stem cell systems and would provide a means for systemic endocrine regulation of the SSC niche. The niche would be more plastic and possibly change location if dependent on vascular and interstitial regulation.

Testosterone regulation of spermatogonia

Androgen receptor (AR) is expressed in testicular somatic cells, Sertoli, Leydig, and peritubular myoid, which may all contribute to the SSC niche (Walker & Cheng 2005, Tsai *et al.* 2006). Multiple genes regulated by testosterone *in vivo* have been identified in the testis (Sadate-Ngatchou *et al.* 2004), but the molecular mechanisms by which testosterone regulates somatic and germ cell function in the testis are unclear. Reports of AR expression in differentiating germ cells have been controversial because of conflicting data on which germ cells express AR. The data indicates that AR expression is present in some differentiating germ cells in certain species. Elongated spermatids have been shown to express AR and the AR-specific co-regulator, SNURF/RNF4 (Vornberger *et al.* 1994, Yan *et al.* 2002). In addition, we have unpublished data that gonocytes express AR during neonatal life in pigs and bulls.

The expression of AR in differentiating germ cells is not required for spermatogenesis because transplantation of germ cells from the naturally occurring AR mutants called testicular feminized (*tfm*) mice into the testes of wild-type mice resulted in complete germ cell differentiation (Johnston *et al.* 2001). These results suggest that germ cells do not need to express AR in order to differentiate into sperm. However, numerous reports have demonstrated that AR expression in Sertoli cells is essential for germ cell differentiation into sperm (De Gendt *et al.* 2004, Holdcraft & Braun 2004).

Testosterone is essential for testis development and sustained spermatogenesis. However, recovery of spermatogonial proliferation is blocked by testosterone following cytotoxic damage to the testis in rats from multiple toxicants including radiation (Meistrich & Shetty 2003, Shetty *et al.* 2006a). In addition, FSH also blocks spermatogonial proliferation independently of testosterone following irradiation. The negative action of FSH and testosterone on spermatogonial proliferation in this model is independent of SSCs and is due to the somatic environment. Radiation does not alter the functional ability of rat SSCs to colonize the

seminiferous tubules of recipient immunodeficient nude mice. The block on spermatogonial differentiation in irradiated rats is actually due to damage to the somatic cells in the testis. This was demonstrated with the use of SSC transplantation by the transplantation of germ cells from irradiated rats into the testes of irradiated or untreated recipient rats (Zhang *et al*. 2007). Suppression of testosterone was necessary for colonized rat SSCs to differentiate in the testes of irradiated rat recipients but testosterone suppression was not required if the rat SSCs were injected into the seminiferous tubules of control recipient rats. Interestingly, transplantation of rat SSCs into the seminiferous tubules of bulsulfan–treated or irradiated mouse testes demonstrated that the colonization of the rat SSCs and subsequent spermatogonial differentiation were not inhibited by the high intratesticular testosterone concentrations in irradiated mice. Thus, the block of spermatogonial differentiation due to high concentrations of FSH and testosterone following irradiation appears to be specific to rats (Zhang *et al*. 2007).

The age–related decline in testosterone is due to testicular aging, and at least 30% of men >60–70 years of age have low testosterone concentrations (Hijazi & Cunningham 2005). Testicular aging may be due to decreased Leydig cell function or changes in the pulsatility of LH leading to a decreased production of testosterone. A similar decline in serum testosterone is observed at 15 months of age in mice, leading to a severe depletion of germ cells and the presence of vacuoles in the seminiferous epithelium (Lacombe *et al*. 2006). Indeed, up to 70% of the seminiferous tubules in 15-month-old mice show degeneration. Ryu *et al*. (2006) showed that SSCs from 12 to 24-month-old mice maintain their activity when transplanted into the testes of young mice suggesting that SSCs are functional past the lifespan of the male. These authors concluded that infertility in old males results from deterioration of the SSC niche and a failure of the niche to support the appropriate balance between stem cell self-renewal and differentiation (Ryu *et al*. 2006). These results, taken with the report that the lack of AR expression in peritubular myoid cells leads to oligospermia (Zhang *et al*. 2006), indicate that testosterone is needed to support the SSC niche and sperm production throughout the lifespan of the male. Likewise, the decrease in serum testosterone levels during aging in males leads to a decrease in the number of SSCs resulting in decreased sperm production.

Conclusions and future directions

The central two questions addressed in this review – what stimulates a SSC to self-renew and what stimulates a SSC to commit to differentiation provide the framework around which regulation of the SSC population is formed. Much is expected of SSCs including maintaining a stable population that can produce progeny cells for the majority of the life of the male. Beyond this function, the homeostasis of the cell population, cellular mechanisms must maintain precise
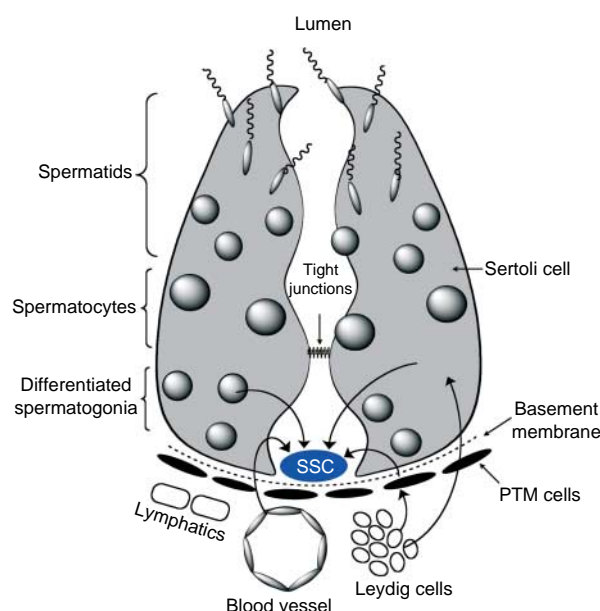

Figure 2 Diagram is a model of spermatogonial stem cell and somatic cells contributing to the SSC niche. This model demonstrates the sources of potential autocrine, paracrine, and environmental cues that may influence spermatogonial stem cell fate in the mammalian testis.

replication of genetic material in SSCs because of the potential downstream contribution to the genome of offspring. Since the SSC is the only adult stem cell population that contributes genetic information to subsequent generations, the requirement of 'stemness' in SSCs may be more complex than other adult stem cells. Clearly defining the stemness of any adult stem cell population will improve our ability to discern the transdifferentiation potential of a cell and if adult stem cells have advantages over differentiated cells in this regard. Although external cues are known to regulate SSCs (Fig. 2), other adult stem cells are directed by intrinsic signals. Potentially intrinsic signals in SSCs may provide the stimulus of a SSC to differentiate with the loss of external signals to maintain stemness.

This review focused primarily on information gained with the use of rodent SSC models. However, translation of these findings to humans or agricultural species requires more comparative research on regulation of SSCs. In contrast to humans, the development of the testis and SSC population in rodents is rapid. Testis development in some large species (e.g. cattle, horses, pigs) falls between the rapid testis development in rodents and the protracted testis development in humans. Investigation of the process of the formation and maintenance of the SSC population in different species will establish if the same mechanisms regulating SSCs in rodents are used in all species and if the process just takes longer or if unique signals are required to maintain future SSCs in a quiescent state. The small number and lack of specific marker for SSCs in the testis

create challenges to investigate the regulation of these cells; however, expansion of our knowledge base about the mechanisms for self-renewal provide the basis for understanding the mechanisms regulating differentiation and the regulation of factors produced by the somatic cells of the niche that influence these processes.

Declaration of interest

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