Role of the testis interstitial compartment in spermatogonial stem cell function

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Abstract

Intricate cellular and molecular interactions ensure that spermatogonial stem cells (SSCs) proceed in a step-wise differentiation process through spermatogenesis and spermiogenesis to produce sperm. SSCs lie within the seminiferous tubule compartment, which provides a nurturing environment for the development of sperm. Cells outside of the tubules, such as interstitial and peritubular cells, also help direct SSC activity. This review focuses on interstitial (interstitial macrophages, Leydig cells and vasculature) and peritubular (peritubular macrophages and peritubular myoid cells) cells and their role in regulating the SSC self-renewal and differentiation in mammals. Leydig cells, the major steroidogenic cells in the testis, influence SSCs through secreted factors, such as insulin growth factor 1 (IGF1) and colony-stimulating factor 1 (CSF1). Macrophages interact with SSCs through various potential mechanisms, such as CSF1 and retinoic acid (RA), to induce the proliferation or differentiation of SSCs respectively. Vasculature influences SSC dynamics through CSF1 and vascular endothelial growth factor (VEGF) and by regulating oxygen levels. Lastly, peritubular myoid cells produce one of the most well-known factors that is required for SSC self-renewal, glial cell line-derived neurotrophic factor (GDNF), as well as CSF1. Overall, SSC interactions with interstitial and peritubular cells are critical for SSC function and are an important underlying factor promoting male fertility.


Defining a spermatogonial stem cell

Mammalian spermatogonial stem cells (SSCs), the resident testicular germline stem cell population, are derived from postnatal quiescent progenitor cells (licensed T2-prospermatogonia). Within the adult testis, SSCs have the unique ability to self-renew or divide into more differentiated progeny (Kluin & de Rooij 1981, Yoshida et al. 2006). SSCs are definitively defined by their stem-like qualities (proliferation, self-renewal and expansion) using functional assays, such as in vivo transplantation, in vitro clonal proliferation and in vitro cobblestone assays; it has proven difficult to determine SSCs precisely in vivo with a single molecular marker, because many of their phenotypic characteristics overlap with their progeny, the A-type undifferentiated spermatogonia (Ploemacher et al. 1989, Brinster & Zimmermann 1994, Dobrinski et al. 1999, Kubota & Brinster 2006, Kanatsu-Shinohara et al. 2012). Classically, SSCs are characterized in many mammalian systems by the expression of a combination of markers, such as cadherin 1 (CDH1), glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRα1), inhibitor of differentiation 4 (ID4), integrins alpha 6 and beta 1, strong expression of zinc finger and BTB domain containing 16 (ZBTB16H), also known as promyelocytic leukemia zinc finger (PLZFHi), ret proto-oncogene (RET) and Thy1, and the lack of kit oncogene (KIT) and stimulated by retinoic acid 8 (STRA8) expression (Meng et al. 2000, Giuli et al. 2002, Kubota et al. 2003, Naughton et al. 2006, Kanatsu-Shinohara et al. 2008, Tolkunova et al. 2009, Sun et al. 2015, Takashima et al. 2015).

Based on the calculated number of A single (single unchained) spermatogonial cells (see below for further discussion of these cells), SSCs represent only about 0.03% of the total mouse germ cell population (up to 35,000 SSCs/testis) (Tegelenbosch & de Rooij 1993). Although they represent only a small portion of the total germ cell population, SSCs are absolutely critical for sustaining sperm production and maintaining long-term fertility by tightly balancing self-renewal and differentiation (i.e., producing spermatogonial progeny). The stem cell niche, comprising testicular somatic cells, provides important extrinsic signals that assist SSCs in determining the balance between self-renewal and differentiation (discussed in later sections). The cell types that release SSC regulatory factors, specifically those cell types located within the testicular interstitial and peritubular compartments, are the major focus of this review.
Models of SSC division

SSCs have dual functions: they can both self-renew and produce differentiated progeny. The renewal function of SSCs requires their distribution across the seminiferous tubule, with different densities based on the staged cohorts of spermatogenesis to continuously produce progeny. In contrast to self-renewal, the transition of SSCs and their immediate progeny, undifferentiated spermatogonia, into differentiated A1 spermatogonia is triggered at a defined stage during the spermatogenic cycle. SSC self-renewal is characterized by three separate, but important, functions: proliferation; survival and a select proportion of cells that do not differentiate, but rather maintain stem cell-like functionality and features.

There are two major theories regarding the mechanism by which SSC population(s) renew or differentiate (Fig. 1). Their differences may be attributed to the different mammalian species from which each model was generated. One model is based on work on rats performed in both Huckins’ and Oakberg’s laboratories, with a hierarchy of A_single (single unchained), A_paired (two-cell chain) and A_aligned (four-cell chain or longer) undifferentiated spermatogonia, whereas the other model is based on several publications by Clermont with others, based on an active (A0) and reserve (A1) non-human primate stem cell pool. In the model developed from Huckins’ and Oakberg’s findings, the key step corresponding to self-renewal, as compared to differentiation, occurs at A_single’s division into A_paired. The mitotic division can result in either a complete cytokinesis, leading to self-renewal to form two separate single cells, or an incomplete cytokinesis, which results in intracellular cytoplasmic bridges that produce a two-cell connected chain of A_paired cells. Further rounds of divisions (all with incomplete cytokinesis) produce chains of up to 16 (or rarely 32) linked A_aligned undifferentiated spermatogonia (Huckins 1971, Oakberg 1971).

In the other model based on non-human primate studies by Clermont and colleagues, cells are classified as A0 and A1, which are distinguishable by their distinct nuclear morphology visualized by hematoxylin staining intensity, defined as A_dark and A_pale respectively (Clermont & Leblond 1953, Clermont & Bustos-Obregon 1968, Dym & Clermont 1970, Clermont & Hermo 1975). The A0 in this model is a quiescent or ‘reserve’ stem cell, which does not divide. Irradiation experiments, in which all dividing cells are ablated, confirm the presence of this normally quiescent cell, which allows for the A0 reserve pool to re-populate the A1 SSCs under certain conditions. Clermont and colleagues claim the active A1 stem cell pool consists of only A1–A4 spermatogonia (corresponding to the A_single, A_paired and A_aligned cells in the other proposed model), and the A4 spermatogonia divide to produce either new A1 spermatogonia for self-renewal or differentiate to produce intermediate spermatogonia.

As further support for the Oakberg/Huckins SSC model, several groups have proposed that cells from spermatogonial chains (i.e., A_paired, A_aligned) can ‘break off’ and give rise to SSC A_single cells (or A_paired cells) both during steady state and after testicular insult, such as irradiation and busulfan treatment. However, other groups have restricted the A_single theory further by stating that the definitive SSC pool is an even smaller subset of cells (only ~3000 cells per mouse testis) which are ID4 positive, whereas ID4-negative cells should be termed ‘committed’ progenitor cells (van Keulen & de Rooij 1975, Lok & de Rooij 1983, Lok et al. 1983, van Beek et al. 1984, Nagano 2003, Nakagawa et al. 2007, 2010, Oatley et al. 2011a, Chan et al. 2014). These ‘progenitors’ can still go through a limited number of mitotic divisions of self-renewal, but are primed to differentiate. What started off as two theories differing in the fact that the stem cell pool is composed of single cells (A_single) or chains of cells, now has become a more complex issue. Further work to elucidate this intricate balance and process is required to understand how to define the ‘true’ stem cell population; only then can we...
have an in-depth understanding of how SSCs control the balance between self-renewal and differentiation.

The SSC niche

The balance between self-renewal and differentiation is not only dependent on SSC intrinsic factors but also requires extrinsic signaling to regulate the process. SSC populations reside within roughly 3000–40,000 germinal testicular stem cell niches (at least within mouse models), where they receive important signals to control their own numbers and to produce differentiated progeny that ultimately result in sperm (Tegelenbosch & de Rooij 1993, Shinohara et al. 2001). The stem cell niche is critical for both maintaining the stem cell population and contributing to the differentiation of daughter cells, which together drive the overall fertility of the organism and simultaneously prevent tumor growth. The interaction of testicular ‘niche’ cells with SSCs occurs via both cellular contact and soluble signaling. Although there are intrinsic factors that contribute to SSC regulation, these extrinsic niche factors are just as important for SSC fate.

To demonstrate the importance of extrinsic signaling for stem cell function, donor SSCs localized within differentially staged tubules were investigated for different levels of stem cell activity or colony expansion upon their transplantation. These studies revealed that donor SSCs obtained from stages IX to IV tubules have higher stem cell function (longer colony length), when compared to donor SSCs from stages V to VIII, linking responsiveness to local gradients of morphogens, including follicle-stimulating hormone (FSH), androgen and retinoic acid (RA), to the level of stem cell activity (Caires et al. 2012a). These gradients are part of the local niche and direct the type of division in a stage-dependent manner (Caires et al. 2012a). The FSH gradient is important at particular spermatogenic stages to drive Sertoli cell release of GDNF for SSC self-renewal. Androgens are important to drive release of GDNF by peritubular myoid cells for SSC self-renewal; during this time, the cell prepares for ‘commitment’ to differentiation (i.e., gains the ability to respond to RA), which is induced upon exposure to RA and results in differentiating the spermatogonia.

Several external factors are known to trigger SSC/undifferentiated spermatogonial division toward differentiation. A wave of testicular retinoic acid (RA) drives spermatogenesis by promoting spermatogonial differentiation and entry into meiosis. In RA depletion models, either by lack of dietary vitamin A or blocking the RA biosynthesis pathway genetically or pharmacologically, germ cells are arrested at the undifferentiated spermatogonial stage (Wolbach & Howe 1925, Mitranond et al. 1979, Griswold et al. 1989, Brooks & van der Horst 2003, Amory et al. 2011, Li et al. 2011), indicating the importance of RA in spermatogenesis. Additionally, the Stra8 gene, important for gametogenesis during the leptotene stage of prophase I in pre-meiotic cells, is induced at stage VI of the spermatogenic cycle (Zhou et al. 2008b). Sertoli cells contribute to the source of RA, which has been shown to influence their own maturation (inducing a quiescent phenotype through inhibiting activin-induced proliferation and influencing tight junction formation through occludin expression), as well as SSC differentiation (Hasegawa & Saga 2012, Nicholls et al. 2013). The impact of RA on SSC differentiation is suggested to be either direct or indirect through Sertoli cells in pre-pubertal mice (first wave of spermatogenesis, from gonocytes) (Raverdeau et al. 2012, Yang et al. 2016). The indirect influence of RA through Sertoli cells is not mediated through Sertoli cell expression of retinoid X receptors (RXRs) (shown by triple-ablation RXRa/β/γ mouse models), but rather solely through RA receptors (RARs) (the other partner of the heterodimer) alone (Vernet et al. 2006, Raverdeau et al. 2012). RA also directly influences SSC decision making by promoting differentiation by increasing Stra8 and Kit and inhibiting self-renewal by reducing POU5F1 (also known as OCT4) and ZBTB16 expression (Dann et al. 2008, Zhou et al. 2008a, Busada et al. 2015, Yang et al. 2016). Ectopic expression of RARγ in GFRA1-positive cells (which normally do not express Kit) allowed for the expression of Kit without passing through the normal neurogenin 3 (NGN3)-positive stage (which normally expresses Kit), demonstrating that functional RA signaling must be acquired through upregulation in ‘committed’ undifferentiated spermatogonia (Ikami et al. 2015).

Other factors can synergize with RA signaling to boost its influences. The autocrine influence of one of these other factors, bone morphogenetic protein 4 (BMP4), through its receptors, BMPR1a and BMPRII, cannot influence differentiation alone, but boosts RA signaling in mouse SSCs in vitro. Additionally, BMP4’s antagonist, Noggin, can prevent RA-induced expression of Stra8 and Kit (Yang et al. 2016).

As defining the niche is elusive, more information is needed regarding the cells that contribute to this microenvironment. Understanding niche signaling is important to determine what controls the balance between SSC self-renewal and differentiation. This review focuses on the interaction of the interstitial and peritubular populations of cells that influence SSC behavior. The mammalian testicular interstitium contains vasculature, Leydig cells, and macrophages, while the peritubular region consists of peritubular myoid cells (PMCs) and macrophages (Fig. 2). All of these cell types will be covered in more detail as we describe factors they release that directly or indirectly influence SSCs.
after transplantation in a similar manner to normal fetal development and after adult SSC transplantation. The transplantation of SSCs into postnatal hosts, before the formation of the blood–testis barrier (BTB), allows for more effective colonization of SSCs as compared to an adult host. SSCs attach to Sertoli cells and transmigrate to the basement membrane of the seminiferous tubules using the laminin receptor (comprised of integrins alpha 6 and beta 1), the chemokine (C-X-C motif) receptor 4 (CXCR4), and RAS-related C3 botulinum substrate 1 (RAC1), a Rho-GTPase, to transmigrate and colonize the niche (Kanatsu-Shinohara et al. 2008, 2012, Takashima et al. 2011).

Upon initial examination of the localization of SSCs, which are numerous in the testis and spread throughout the organ, it might seem as if SSCs are randomly distributed within seminiferous tubules. However, systematic and morphometric analyses have revealed a non-random distribution of SSCs within seminiferous tubules; in particular, studies have focused on whether SSCs are preferentially localized to regions of the tubules that directly contact interstitial cells on the other side of the tubule basement membrane vs direct contact with other tubules without intervening interstitial tissue. Chiarini-Garcia and colleagues described niche positioning changes during development in both rats and mice (Chiarini-Garcia et al. 2001, 2003). Upon SSC transplantation, subsequent SSC movement and distribution around the tubule resulted in an observed asymmetry of SSC localization to interstitial-associated regions (areas rich in vasculature, Leydig cells and macrophages). This localization to the interstitial region is seemingly in a stage-specific manner (stages IV–VI). To build upon this interstitial–SSC association concept, do Nascimento and colleagues demonstrated that hamster SSCs localize to the adjacent interstitial region, rather than juxtaposing tubules, during the active mating season in a photoperiod-dependent manner (do Nascimento et al. 2009).

The driving factors and cell types involved in niche localization are still unclear. In adult mice, Yoshida and colleagues demonstrated that undifferentiated spermatogonia are associated with interstitial areas (Yoshida et al. 2007). Using antibodies targeting three proteins (GFRA1, CSF1R and PLZF/ZBTB16) expressed in undifferentiated spermatogonia, it was determined that undifferentiated spermatogonia preferentially localize near the interstitium in equine species, such as stallions and mules (Costa et al. 2012). In the collared peccary (Tayassu tajacu), the Leydig cells form lobes, which allow a way to analyze the specific influence of Leydig cells (apart from the rest of the interstitium) upon the position of SSCs. Interestingly, 93% of undifferentiated spermatogonia, as marked by GFRA1, are located near the interstitium, rather than oriented toward either the Leydig lobes or adjacent seminiferous tubules; therefore, macrophages and vasculature may

**SSC colonization to the niche**

The homing ability of SSCs to the niche is important for establishing spermatogenesis. Niche factors that promote SSC division are just as important as the niche factors required for the colonization of SSCs to the niche, because reduced or absent colonization leads to impaired fertility. After birth, during normal conditions in mice, gonocytes migrate from the center of the seminiferous tubule to the outer (basal) edge of the seminiferous tubule. Transplantation studies utilizing SSCs defective in particular genes have helped reveal important mechanisms in SSC recruitment and localization. Microinjection of SSCs into the seminiferous tubules or efferent ducts for transplantation studies simulate normal developmental transmigration, as primordial germ cells (PGCs) isolated from as early as 13.5 days post coitum can colonize adult seminiferous tubules.
some groups speculate that either the interstitium or Sertoli cells supply the directing signals, whereas others postulate that the combination of vasculature, which brings in FSH to the local area, and Sertoli cells, which are activated by FSH and subsequently produce glial cell line-derived neurotrophic factor (GDNF), together are important for SSC localization (Yoshida et al. 2007, De Rooij 2009, Oatley et al. 2011b). Although Sertoli cells and the basement membrane of the seminiferous tubules are in direct contact with SSCs, additional interstitial and peritubular secreted signals may be required to maintain SSCs within the stem cell niche. We will now discuss the potential contribution of each interstitial/peritubular cell type to drive SSC function in more detail (Fig. 3).

**Interaction with interstitial/peritubular cells**

**Leydig cells**

Leydig cells, upon luteinizing hormone (LH) stimulation, regulate the expression level of steroidogenic enzymes, such as 17-β hydroxysteroid dehydrogenase, to increase the production of testosterone. Testosterone is known to influence spermatogenesis in several ways, and can either bind the androgen receptor (AR) to exert its effects locally within the testis or can bind to androgen-binding
protein (ABP), which allows higher levels of testosterone in the seminiferous tubules and its transport to the epididymis (Smith & Walker 2014). AR is expressed in Sertoli cells, PMCs, Leydig cells and spermatids within the testes (Sar et al. 1990, Takeda et al. 1990). One way in which testosterone (or a testosterone derivative, dihydrotestosterone) exerts its influence is by binding to cytoplasmic AR, which releases AR from the cytoplasm, translocates it to the nucleus and allows for its DNA-binding transcription factor function (such as its regulation of the transcription of Igf1r, encoding IGF1R, an important receptor for signaling by another Leydig cell factor, IGF1; discussed later in this section). All cell types have their own regulation of nuclear AR expression based on their receptivity to androgen levels (Blok et al. 1992, Zhu et al. 2000). None of the germ cell subsets in the adult, besides round and elongating spermatids, expresses AR; however, fetal gonocytes do express Ar and functional AR protein, which is thought to restrict their proliferation and induce their fetal quiescence (Merlet et al. 2007).

Within the adult, the influence of testosterone is exerted as an indirect effect through Sertoli-cell-controlled attachment mechanisms and via PMC-secreted factors (discussed in ‘Peritubular myoid cells’ section). Analyses of Ar-deficient testes revealed that testosterone drives several adhesion functions for Sertoli cell interactions, including Sertoli–Sertoli adhesions to create the BTB (comprising junctional molecules such as occludin, claudin 3 and claudin 11), thus providing protection for cells transitioning through meiosis; and Sertoli–germ cell adhesions, for ability of stage VII and VIII spermatids to attach to Sertoli cells and for the release of mature sperm from Sertoli cells into the tubule lumen (Yeh et al. 2002, Chang et al. 2004, De Gendt et al. 2004, Holdcraft & Braun 2004, Meng et al. 2005, Wang et al. 2006, Walker 2010).

One report that demonstrates the influence of mouse testosterone on SSCs is indirectly through modulation of Sertoli cells via wingless-type MMTV integration site family, member 5A (WNT5A). However, 7-day-old postnatal testes had higher levels of WNT5A as compared to the 8-week-old adult; therefore, it does not seem that WNT5A expression correlates with the normal ‘steady-state’ SSC population or the ‘peak’ of testosterone production. The evidence that testosterone indirectly influences SSCs is through the use of the LH/choriogonadotropin receptor (Lhcgr)-deficient mouse. This mouse lacks the ability to functionally respond to LH and, thus, cannot effectively produce testosterone and has undescended testes (resulting from reduced secretion of insulin-like 3, which is required for testicular descent) (Tanaka et al. 2016). Lhcgr-deficient mice display an immature Sertoli cell phenotype, which is required for Sertoli cells to express high levels of WNT5A and subsequently influence SSCs; however, analysis of Sertoli cell maturation was based upon maturation markers that were testosterone driven, but testosterone was lacking in this model (Tanaka et al. 2016). If Sertoli cell maturation is analyzed based on the expression of non-testosterone-driven Sertoli maturation markers, such as keratin 18 and antigen identified by monoclonal antibody Ki 67 (also known as MKI67 and commonly referred to as ki-67) expression (both of which appeared unaltered), the Sertoli cells actually have a ‘mature’ phenotype (Tanaka et al. 2016). These data and data from when the authors ‘normalized’ testes using germ cell depletion via busulian treatment (which removes germ cells and any differences caused by germ cell-specific changes) and the removal of the influence of testosterone via flutamide (which blocks testosterone-induced AR signaling) confirm that testosterone signaling is the specific event that influences the release of WNT5A, rather than the ‘maturation status’ of Sertoli cells. This alternative experiment addressed concerns that undescended Lhcgr-deficient testes influence SSCs (and other germ cell types) negatively due to other variables, such as changes in the microenvironment (e.g., temperature increase due to undescended testes), rather than the mutation itself (Lei et al. 2001, Zhang et al. 2001, Tanaka et al. 2016). Taking these factors into account, it appears that the mechanism by which testosterone directly exerts influence on SSCs appears to be through AR signaling, but this topic warrants further research.

IGF1 is important for stimulating proliferation in multiple cell types within the testis (including Leydig cells, Sertoli cells, differentiated spermatogonia and SSCs), all of which express IGF1R (Dubois & Callard 1993, Moore & Morris 1993, Zhou & Bondy 1993, Tajima et al. 1995). Within the testis, Leydig cells are a source of insulin-like growth factor 1 (IGF1) and release IGF1 in culture for their own differentiation/maturation, proliferation and testosterone production (Huang et al. 2009). IGF1, when added to SSC cultures maintained by GDNF, increases the proliferation of SSCs; however, the mitogenic effects of IGF1 are only achieved in concert with GDNF, as any beneficial proliferative and survival effects do not occur with IGF1 alone. The mechanism proposed for IGF1 is that it signals through IGF1R and AKT to stimulate DNA synthesis and proliferation at the G1/M checkpoint, which is different from Sertoli cell-released factors, such as GDNF and fibroblast growth factor 2 (FGF2), which signal through MAP kinases MAPK1/3 (also known as ERK1/2) to influence the G1/S transition (Kubota et al. 2004, Wang et al. 2015).

Leydig cells are reported to be one of the several sources of colony-stimulating factor 1 (CSF1), which controls SSC self-renewal (Oatley et al. 2009). CSF1, when supplemented to GDNF/FGF2-maintained cultures, increased the numbers of SSCs within Thy1-positive germ cells (Oatley et al. 2009). Colony-stimulating factor 1 receptor (CSF1R), the receptor for CSF1, is enriched in the SSC/undifferentiated spermatogonial population (Kokkinaki et al. 2009, Oatley et al. 2009).
CSF1, through CSF1R signaling, stimulates proliferation and self-renewal of ex vivo-cultured spermatogonia (Kokkinaki et al. 2009, Oatley et al. 2009); however, further in vivo studies must be performed to determine if CSF1/CSF1R signaling is required for SSC colonization, proliferation or self-renewal in vivo.

**Vasculature**

Vasculature and perivascular cells play a role in both stem cell behavior and localization in multiple systems, including bone marrow (reviewed in Doan & Chute 2012). Data from several groups lead to a proposed model in which Id4-positive A\textsubscript{single} cells localize based on their affinity for avascular areas. These Id4-positive A\textsubscript{single} cells, upon their ‘commitment’ to become spermatogonia (i.e., the combination of a cell division forming A\textsubscript{paired} cells and/or the new expression of Ngn3, resulting in Ngn3-positive A\textsubscript{paired} cells), then re-locate to vascular areas to fulfill their new requirements for different levels of oxygen, metabolites and various growth factors (Yoshida et al. 2007, Chan et al. 2014, DeFalco et al. 2015). The relocation of germ cells within the tubule is also discussed in the ‘SSC colonization to the niche’ section.

One factor thought to be associated with vasculature, and is important for testis-specific vascular remodeling during fetal stages, is vascular endothelial growth factor A (VEGFA). VEGFA is most well-known for its critical roles in endothelial cell proliferation, survival, migration and permeability; however, other non-endothelial cell types have been observed to express VEGF receptors, such as macrophages. There are different isoforms of VEGFA, some of which are pro-angiogenic, whereas others are anti-angiogenic, thereby increasing the complexity of this signaling pathway (Nowak et al. 2008). The anti-angiogenic VEGF isoform 165b (VEGF-165b), preferentially localized within bovine and rodent spermatogonia, is thought to play a role in germ cell survival and differentiation, whereas the pro-angiogenic VEGF-164 isoform is thought to be involved in self-renewal (Caires et al. 2009, 2012b, Lu et al. 2013). The source of VEGF within the adult testis is thought to be from Leydig and Sertoli cells, as Vegfa mRNA is detected, but not translated into VEGFA protein, within germ cells (Caires et al. 2009, Lu et al. 2013). Within adult rodent testes, Leydig cell stimulation by the LH analog human chorionic gonadotropin (hCG) induces the production of VEGFA. The influence of hCG-released VEGFA on vascular endothelial cell proliferation and permeability is dependent on the VEGF receptor KDR (also known as VEGFR2), but macrophage recruitment is a KDR-independent process (Rudolfssson et al. 2004). Within postnatal testes, VEGF receptor expression is differentially expressed within germ cell subsets. FLT1 (also known as VEGFR1) and KDR are expressed in spermatids and spermatogonia respectively; therefore, these receptors may exert differential influences on distinct germ cell populations (Nalbandian et al. 2003). Interestingly, within the kidney, when VEGFA binds KDR, it induces phosphorylation of RET (at tyrosine 1062), providing a cross-talk effect between GDNF and VEGF signaling (GDNF signaling discussed more in detail in ‘Peritubular myoid cells’ section); in this fashion, VEGF signaling may contribute to SSC self-renewal (Tufro et al. 2007).

Although VEGF signaling components (either VEGFA or its receptors) may not be expressed in the vasculature or exert any influence on the vasculature in the adult testis (Lu et al. 2013), a known factor, CSF1, is released from the perivascular compartment (from PECAM1-negative, ACTA2-positive perivascular smooth muscle cells) (DeFalco et al. 2015). CSF1 potentially modulates adult spermatogonial behavior (see further discussion of how CSF1 alters SSC balance in the ‘Leydig cells’ section) (DeFalco et al. 2015).

**Testicular macrophages**

The role of testicular macrophages within the SSC niche is not well understood, although the prominent presence of macrophages within the interstitial compartment, along with their diverse biological functions, has led to speculation that they potentially influence SSCs. Macrophages could indirectly influence SSCs through various mechanisms. One potential effect of macrophages on SSCs is indirectly routed through Leydig cells (see ‘Leydig cells’ section for more detail on Leydig–SSC testosterone interplay), in which macrophages produce an intermediate compound within the testosterone biosynthetic pathway (25-hydroxycholesterol), thus potentially speeding up the testosterone production process (Hutson 1992, Nes et al. 2000). Additionally, intercytoplasmic digitations have been reported between macrophages and Leydig cells. Although the function of the intercytoplasmic digitations is unclear, they reportedly assist in macrophage regulation of Leydig intracellular and mitochondrial ultrastructure required for Leydig cell steroid biogenesis and could potentially serve for transfer of cellular factors, like 25-hydroxycholesterol, between testicular macrophages and Leydig cells (Gaytan et al. 1994, Cohen et al. 1996, 1997, Hutson 2006).

Although macrophages clearly play at least some role in testosterone production, macrophage influence on SSCs most likely is not due to changes in testosterone levels, but potentially other direct and indirect factors, as the testosterone concentration does not fall below the threshold required for spermatogenesis upon macrophage ablation (DeFalco et al. 2015). Under short-term macrophage-depletion conditions, both serum and intra-testicular testosterone levels are sustained above the testosterone threshold (<20% of normal intra-testicular testosterone values) required for quantitatively
and qualitatively maintaining normal spermatogenesis (Awoniyi et al. 1989, Zirkn et al. 1989, DeFalco et al. 2015). Furthermore, these decreases in testosterone levels do not indirectly impact fertility through Sertoli cells, because the blood–testis barrier is still intact (DeFalco et al. 2015). Due to these results, DeFalco and colleagues proposed more direct methods of interaction between macrophages and SSCs, such as via the CSF1 and retinoic acid (RA) pathways, as these factors or components of their biosynthetic pathways are expressed in macrophages (DeFalco et al. 2015), although these claims have not been functionally investigated as of yet. As macrophages potentially secrete many factors, including cytokines, further experiments are needed to determine definitively which macrophage factors and mechanisms are involved in SSC regulation.

RA secretion from Sertoli cells is important mechanistically for SSC differentiation. DeFalco and colleagues have demonstrated that other cells, such as macrophages, also express the RA machinery, although the functional relevance of macrophage-mediated RA production in the testis is unknown. The RA synthesis enzyme, aldehyde dehydrogenase family 1, subfamily A2 (ALDH1A2; also called retinol dehydrogenase 2 (RALDH2)), is expressed within the interstitial testicular macrophage subset, whereas the other RA synthesis enzyme, retinol dehydrogenase 10 (RDH10), is expressed mostly in peritubular macrophages and a limited number of interstitial macrophages, indicating macrophages may be potential SSC niche players through their participation in RA signaling (DeFalco et al. 2015) (see ‘The SSC niche’ section). Furthermore, the importance of macrophages via diphtheria toxin-mediated genetic ablation experiments demonstrated that macrophages likely influence spermatogonial differentiation, but not SSC maintenance (DeFalco et al. 2015). Changes in SSC differentiation (reductions in CDH1-positive chains), as well as reductions in both undifferentiated spermatogonia and A1–A2, differentiating spermatogonia indicate that macrophages may have roles in both differentiation and proliferation (DeFalco et al. 2015).

Macrophages may contribute to proliferation/self-renewal through secretion of CSF1 (see discussion of CSF1 in ‘Leydig cells’ section). Macrophages in other systems/tissues are known to be recruited and to recruit other cells via CSF1/CSF1R mechanisms. Therefore, the influence of macrophage release of CSF1 or the expression of CSF1R may have different functions besides its contribution to the proliferation of SSCs. Additional chemotactic factor-encoding genes, such as Ccl2, Ccl3, Ccl7, Csf1r, Cxcl2, Cxcl14 and Itgal, are enriched in both resident testicular macrophages and SSCs, suggesting that SSCs may be recruited to the niche in a similar fashion to testicular macrophages (Kokkinaki et al. 2008, Oatley et al. 2009, DeFalco et al. 2015). The requirement of vascular and macrophage CSF1 signaling for SSC maintenance and/or regulation has not been functionally assessed and, therefore, is an area of research that warrants further studies.

**Peritubular myoid cells**

Some of the main functions of PMCs, the smooth muscle layer surrounding the tubules, include providing structural support and peristaltic action. However, cellular interaction seems to be another important function. One mechanism for PMCs to maintain SSCs is through the combined action of the AR and GDNF signaling pathways (Spinnler et al. 2010, Chen et al. 2014). PMCs secrete GDNF upon testosterone–AR binding, as demonstrated through in vivo Ar conditional knockout studies, in vitro stimulation cultures and transplantation studies (Chen et al. 2016). GDNF signaling is somewhat controversial, as GFRA1 is not expressed in all possible SSCs, as 10% of A single cells lack GFRA1 and its expression might be age dependent, e.g., postnatal vs adult. Two additional issues that arise with GFRA1 analyses are that the expression of GFRA1 is transient in transplantation assays and that GFRA1-negative SSCs are able to still colonize after transplantation in particular situations; nevertheless, GDNF is the most common growth factor used in SSC culture to maintain self-renewal capability (Ebata et al. 2005, Hofmann et al. 2005, Grisanti et al. 2009). GDNF regulation is likely important because PMCs only produce GDNF during a particular time window (stages II–IV), compared to Sertoli cell production (stages IX–I). Briefly, GDNF binds its receptor GFRA1 on SSCs, followed by a subsequent signaling of a co-receptor RET, a transmembrane receptor tyrosine kinase, within all undifferentiated spermatogonia, which leads to upregulation in Src family kinase (SKF) signaling and activation of genes encoding key transcription factors (e.g., B cell CLL/lymphoma6, member B (Bcl6b), brachyury, Id4, et variant gene 5 (Etv5) and LIM homeobox protein 1 (Lhx1)), leading to self-renewal (Fig. 3) (Sariola & Saarma 2003). GDNF signaling is essential for SSC self-renewal, as knockouts in GDNF pathway components, including Gdnf, Ret or Gfra1, all lose spermatogonia and become infertile, whereas overexpression of Gdnf results in the accumulation of SSCs and no differentiation occurs (Meng et al. 2000, Jain et al. 2004, Naughton et al. 2006, Jiwiwa et al. 2008). Furthermore, disruption of the critical transcription factors in this pathway, such as RNAi-mediated knockdown of Bcl6b, Etv5 and Lhx1 in culture, impairs SSC proliferation (Oatley et al. 2006, 2007). Confirmation of this effect is demonstrated by results that show upstream SKF signaling influences self-renewal through proliferation, but does not affect survival (Oatley et al. 2007). An additional role of GDNF is that it inversely regulates the expression of NGN3, found to be expressed mostly in the ‘committed’ progenitor and differentiated spermatogonial stages (although there has been a mixture of NGN3-positive and
NGN3-negative expression reported in ‘non-committed’ SSCs, so perhaps NGN3 is turned on during the transition to a ‘committed’ state). In contrast, the signal transducer and activator of transcription 3 (STAT3), when activated by cytokines, positively regulates NGN3. Therefore, SSCs with either loss of STAT3 or NGN3 are unable to differentiate (Oatley et al. 2010, Kaucher et al. 2012).

PMCs can contribute to the SSC niche, similar to Sertoli cells, in that the production of GDNF is important for the colonization and maintenance of SSCs within the niche; however, it is not the only potential influence that PMCs have on SSCs. Two studies have reported expression of CSF1 in PMCs (Oatley et al. 2009, DeFalco et al. 2015); therefore, PMCs may use CSF1/CSF1R signaling to regulate SSC activity (see ‘Leydig cells’ section).

**Future perspectives and concluding remarks**

Studies performed within the field tend to focus on understanding the influence of one cell type upon another cell type within the same compartment, e.g., examining the role of macrophages on Leydig cells (both of which are within the interstitial compartment) or the role of Sertoli cells on germ cells (both of which reside in the seminiferous tubule compartment), whereas less research has been dedicated to elucidating the influence of cells across the two different compartments. This type of focus on one compartment at a time is a major reason why we know more about factors and pathways regulating Sertoli cell–germ cell interaction, while our knowledge of the role of interstitial cells on SSCs is relatively limited.

The compartment-specific approach commonly used within the field stresses the idea that Sertoli cells are the major cell type involved in the control of SSCs, due to their proximity and their ‘nurse-like’ qualities; however, with the advances in our understanding of spermatogenesis, it has now become clear that the SSC niche receives input from cells residing outside the tubules, including from the interstitial and peritubular regions. Signaling from the interstitial and peritubular regions provide appropriate cues that influence particular stages of spermatogenesis, either directly acting upon the germ cells or indirectly influencing germ cells through Sertoli cells. Currently the dogma is shifting toward a view that cells throughout the whole testicular environment, including peritubular and interstitial cells, act through paracrine signaling and are essential to maintain proper spermatogenesis.

Many of the testicular cell types have potentially overlapping functions (e.g., different cell types secreting the same factors), which allow for fail-safe mechanisms to maintain fertility by compensating or correcting for when genetic or environmental influences may perturb the normal testicular environment. These overlapping functions are important for biologically maintaining fertility; however, this overlapping functionality of different cell types makes it potentially difficult to determine the influence of a particular interstitial cell type on SSC differentiation and self-renewal independent of other parts of the testicular system. The development of either ex vivo or in vitro organ culture systems will be an important tool to elucidate further the complex signaling pathways involved in the SSC niche, such as RA and CSF1, which are not functionally well defined in the context of SSCs. These culture systems will allow researchers to pinpoint important cell type-specific factors on the SSC niche while avoiding confounding secondary effects from other cell types. These techniques will allow researchers to determine more definitively the role of an individual cell type’s and/or a particular signaling molecule’s contribution to the niche. In particular, the study of testicular macrophages, which are not well understood in the context of SSCs but may have multiple and diverse roles in the testis, will benefit from new techniques. Finally, the increased use of CRISPR and other gene-editing tools, along with next-generation sequencing at the cell-type-specific and single-cell levels, will allow us to have a greater understanding of the functional role of interstitial cells in the SSC niche and, ultimately, improve targeted therapies for male infertility.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality in this review.

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