REPRODUCTION
REVIEW

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.

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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 (GFRA1), inhibitor of differentiation 4 (ID4), integrins alpha 6 and beta 1, strong expression of zinc finger and BTB domain containing 16 ($ZBTB16^{HI}$; also known as promyelocytic leukemia zinc finger ($PLZF^{HI}$)), 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, Giuili *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.

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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 A_1 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 (A_1) and reserve (A_0) 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}. This 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).

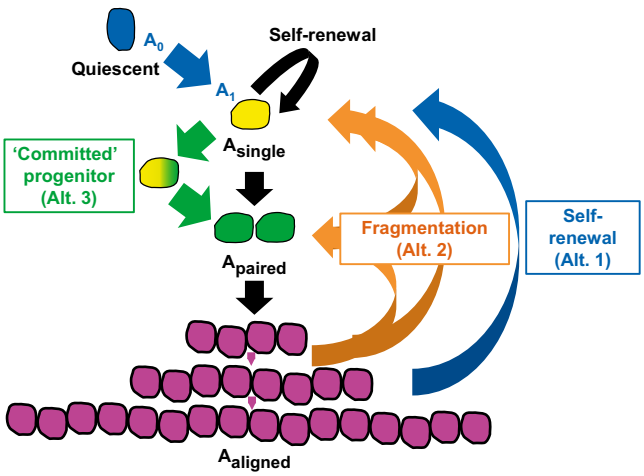

Figure 1 Proposed models of SSC self-renewal. The most accepted model of SSC self-renewal and differentiation (Huckins/Oakberg model) is depicted by black arrows. A subset of A_{single} cells are proposed to be 'true' self-renewing SSCs. These cells divide to give rise to A_{paired} and $A_{aligned}$ undifferentiated spermatogonia. The major alternative model (Alt. 1), proposed by Clermont, is that the A_1 cells (A_{single}, A_{paired}, and $A_{aligned}$) cycle, resulting in long chains of cells that can self-renew (back to A_{single}) or differentiate into differentiated spermatogonia. Part of this proposed model is that there are quiescent (non-dividing) A_0 cells that, under certain conditions, give rise to A_1 cells. The alternative 2 (Alt. 2) model proposes that, during the progression from A_{single} to A_{paired} or A_{paired} to $A_{aligned}$, a few cells can break off to create new A_{single} or new A_{paired} cells. A third alternative model (Alt. 3) proposes that A_{single} cells self-renew or undergo programming to become a 'committed progenitor.' In the 'committed progenitor' stage, an A_{single} is committed to differentiation and upon limited divisions gives rise to A_{paired} undifferentiated spermatogonia.

In the other model based on non-human primate studies by Clermont and colleagues, cells are classified as A_0 and A_1, 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 A_0 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 A_0 reserve pool to re-populate the A_1 SSCs under certain conditions. Clermont and colleagues claim the active A_1 stem cell pool consists of only A_1–A_4 spermatogonia (corresponding to the A_{single}, A_{paired} and $A_{aligned}$ cells in the other proposed model), and the A_4 spermatogonia divide to produce either new A_1 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.* 2011*a*, 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 germline 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.* 2012*a*). These gradients are part of the local niche and direct the type of division in a stage-dependent manner (Caires *et al.* 2012*a*). 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 pharmaceutically, 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.* 2008*b*). 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 RXRα/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.* 2008*a*, 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, BMPRIa 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 *Stra*8 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.

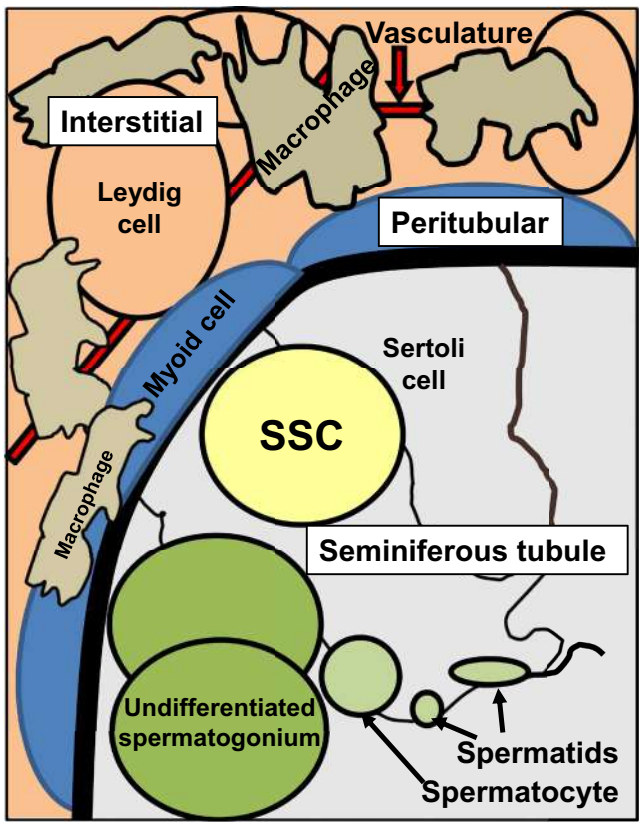

Figure 2 Overview of mammalian testicular structure. This image represents a high-magnification cross-section of an adult mouse testis. Regions are labeled by white boxes as follows: interstitial, peritubular and seminiferous tubule regions. The interstitial region contains the following cell types: Leydig cells (peach), macrophages (brown) and vasculature (red). The peritubular region is composed of myoid cells (blue) and macrophages (brown). The interstitial and peritubular regions are separated from the tubular compartment by a basement membrane (thick black line). The seminiferous tubules contain Sertoli cells (gray), spermatogonial stem cells (SSC, yellow) and later-stage germ cells (different shades of green; not drawn to scale).

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 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

play a major role in SSC localization in this species (Campos *et al.* 2012).

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.* 2011*b*). 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

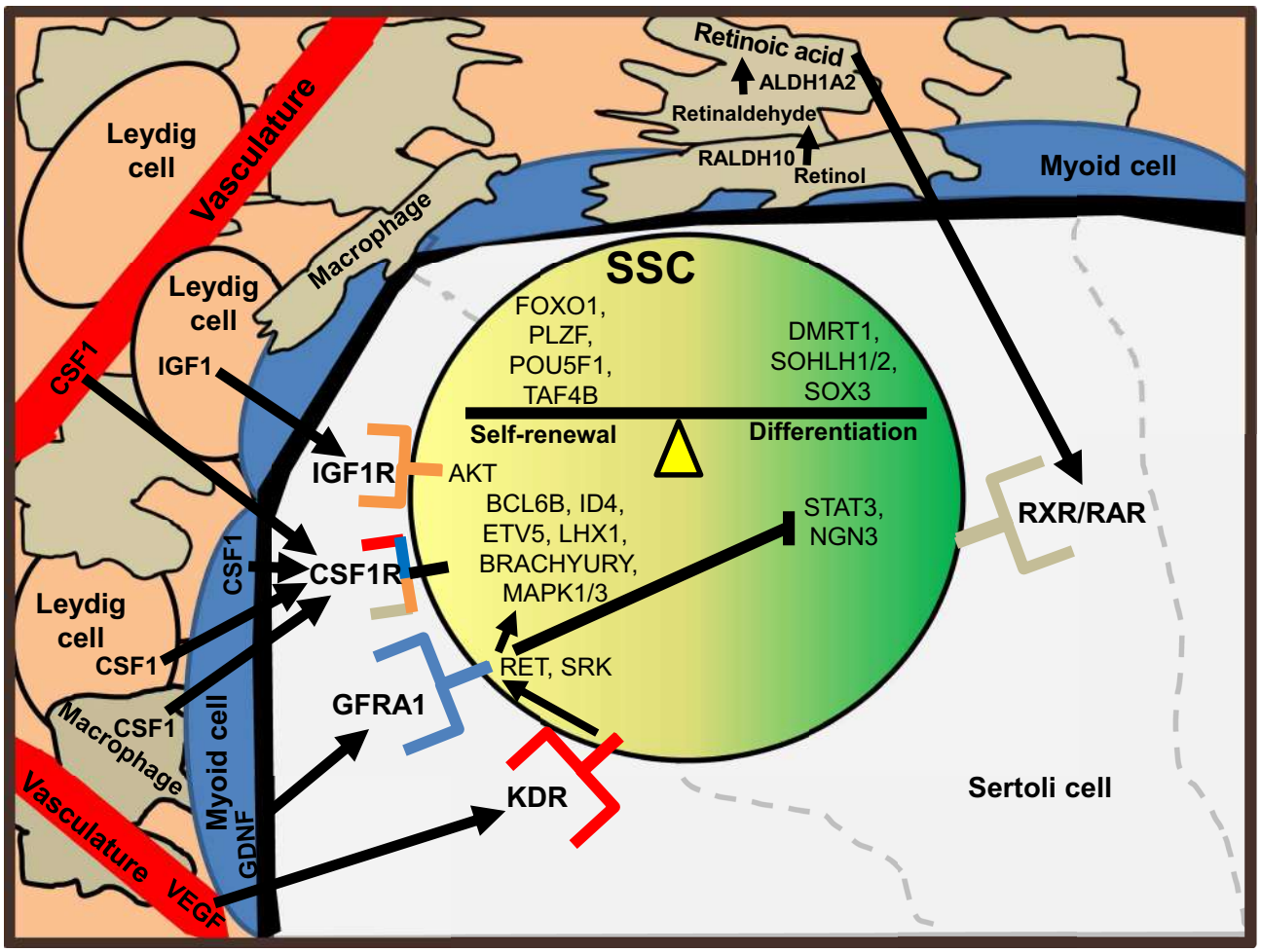

Figure 3 Interstitial/peritubular contributions to the SSC niche. The interstitial region contains the following cell types: Leydig cells (peach), macrophages (brown) and vasculature (red). The peritubular region is composed of myoid cells (blue) and macrophages (brown). For either self-renewal or differentiation, external signals influence SSC fate. Intrinsic germ cell factors promoting self-renewal are found within the yellow (left) side of the SSC, whereas factors that promote differentiation are found on the green (right) side of the SSC and are placed on the respective sides of the balance within the SSC. Extrinsic cues that regulate the niche are depicted by arrows; their receptors are localized on the yellow side of the SSC if they regulate self-renewal or the green side if they regulate differentiation. IGF1R, CSF1R, GFRA1 and KDR all promote self-renewal (yellow), whereas the RXR/RAR receptor complex promotes the transition to undifferentiated spermatogonia (non-self-renewal capability). The colors of receptors are based on the cell type from which the signal is provided (e.g., IGF1 signals are from Leydig cells to promote SSC proliferation; therefore, the receptor is peach colored). The factors are listed within the cells from which they are secreted.

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 busulfan 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 G_2/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 G_1/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_{single} cells localize based on their affinity for avascular areas. These *Id4*-positive A_{single} cells, upon their 'commitment' to become spermatogonia (i.e., the combination of a cell division forming A_{paired} cells and/or the new expression of *Ngn3*, resulting in *Ngn3*-positive A_{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, 2012*b*, 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 (Rudolfsson *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, Zirkin *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 A_1–A_3 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*, *Cxcl4* 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.* 2009, 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 (SFK) signaling and activation of genes encoding key transcription factors (e.g., *B cell CLL/lymphoma6, member B* (*Bcl6b*), *brachyury*, *Id4*, *ets 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, Jijiwa *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 SFK 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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References

Amory JK, Muller CH, Shimshoni JA, Isoherranen N, Paik J, Moreb JS, Amory DW Sr, Evanoff R, Goldstein AS & Griswold MD 2011 Suppression of spermatogenesis by bisdichloroacetyldiamines is mediated by inhibition of testicular retinoic acid biosynthesis. *Journal of Andrology* **32** 111–119. (doi:10.2164/jandrol.110.010751)

Awoniyi CA, Santulli R, Sprando RL, Ewing LL & Zirkin BR 1989 Restoration of advanced spermatogenic cells in the experimentally regressed rat testis: quantitative relationship to testosterone concentration within the testis. *Endocrinology* **124** 1217–1223. (doi:10.1210/endo-124-3-1217)

Blok LJ, Themmen AP, Peters AH, Trapman J, Baarends WM, Hoogerbrugge JW & Grootegoed JA 1992 Transcriptional regulation of androgen receptor gene expression in Sertoli cells and other cell types. *Molecular and Cellular Endocrinology* **88** 153–164. (doi:10.1016/0303-7207(92)90020-7)

Brinster RL & Zimmermann JW 1994 Spermatogenesis following male germ-cell transplantation. *PNAS* **91** 11298–11302. (doi:10.1073/pnas.91.24.11298)

Brooks NL & van der Horst G 2003 Short-term effects of N′N-bis(dichloroacetyl)-1,8-octamethylenediamine (WIN 18446) on the testes, selected sperm parameters and fertility of male CBA mice. *Lab Animal* **37** 363–373. (doi:10.1258/002367703103051921)

Busada JT, Chappell VA, Niedenberger BA, Kaye EP, Keiper BD, Hogarth CA & Geyer CB 2015 Retinoic acid regulates Kit translation during spermatogonial differentiation in the mouse. *Developmental Biology* **397** 140–149. (doi:10.1016/j.ydbio.2014.10.020)

Caires KC, de Avila J & McLean DJ 2009 Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis. *Reproduction* **138** 667–677. (doi:10.1530/REP-09-0020)

Caires KC, de Avila J & McLean DJ 2012*a* Endocrine regulation of spermatogonial stem cells in the seminiferous epithelium of adult mice. *BioResearch Open Access* **1** 222–230. (doi:10.1089/biores.2012.0259)

Caires KC, de Avila JM, Cupp AS & McLean DJ 2012*b* VEGFA family isoforms regulate spermatogonial stem cell homeostasis in vivo. *Endocrinology* **153** 887–900. (doi:10.1210/en.2011-1323)

Campos PHA, Costa GMJ, Lacerda SMSN, Rezende-Neto JV, de Paula AM, Hofmann MC & de Franca LR 2012 The spermatogonial stem cell niche in the collared peccary (Tayassu tajacu). *Biology of Reproduction* **86** 155. (doi:10.1095/biolreprod.111.095430)

Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS & Oatley JM 2014 Functional and molecular features of the Id4+ germline stem cell population in mouse testes. *Genes and Development* **28** 1351–1362. (doi:10.1101/gad.240465.114)

Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, Guillou F, Lardy H & Yeh S 2004 Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. *PNAS* **101** 6876–6881. (doi:10.1073/pnas.0307306101)

Chen LY, Brown PR, Willis WB & Eddy EM 2014 Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. *Endocrinology* **155** 4964–4974. (doi:10.1210/en.2014-1406)

Chen LY, Willis WD & Eddy EM 2016 Targeting the Gdnf gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. *PNAS* **113** 1829–1834. (doi:10.1073/pnas.1517994113)

Chiarini-Garcia H, Hornick JR, Griswold MD & Russell LD 2001 Distribution of type A spermatogonia in the mouse is not random. *Biology of Reproduction* **65** 1179–1185. (doi:10.1095/biolreprod65.4.1179)

Chiarini-Garcia H, Raymer AM & Russell LD 2003 Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. *Reproduction* **126** 669–680. (doi:10.1530/rep.0.1260669)

Clermont Y & Bustos-Obregon E 1968 Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted 'in toto'. *American Journal of Anatomy* **122** 237–247. (doi:10.1002/aja.1001220205)

Clermont Y & Hermo L 1975 Spermatogonial stem cells in the albino rat. *American Journal of Anatomy* **142** 159–175. (doi:10.1002/aja.1001420203)

Clermont Y & Leblond CP 1953 Renewal of spermatogonia in the rat. *American Journal of Anatomy* **93** 475–501. (doi:10.1002/aja.1000930308)

Cohen PE, Chisholm O, Arceci RJ, Stanley ER & Pollard JW 1996 Absence of colony-stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice results in male fertility defects. *Biology of Reproduction* **55** 310–317. (doi:10.1095/biolreprod55.2.310)

Cohen PE, Hardy MP & Pollard JW 1997 Colony-stimulating factor-1 plays a major role in the development of reproductive function in male mice. *Molecular Endocrinology* **11** 1636–1650. (doi:10.1210/mend.11.11.0009)

Costa GMJ, Avelar GF, Rezende-Neto JV, Campos PHA, Lacerda SMSN, Andrade BSC, Thome RG, Hofmann MC & Franca LR 2012 Spermatogonial stem cell markers and niche in equids. *PLoS ONE* **7** e44091. (doi:10.1371/journal.pone.0044091)

Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL & Porteus MH 2008 Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. *Stem Cells* **26** 2928–2937. (doi:10.1634/stemcells.2008-0134)

De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C *et al*. 2004 A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *PNAS* **101** 1327–1332. (doi:10.1073/pnas.0308114100)

De Rooij DG 2009 The spermatogonial stem cell niche. *Microscopy Research and Technique* **72** 580–585. (doi:10.1002/jemt.20699)

DeFalco T, Potter SJ, Williams AV, Waller B, Kan MJ & Capel B 2015 Macrophages contribute to the spermatogonial niche in the adult testis. *Cell Reports* **12** 1107–1119. (doi:10.1016/j.celrep.2015.07.015)

do Nascimento HF, Drumond AL, de Franca LR & Chiarini-Garcia H 2009 Spermatogonial morphology, kinetics and niches in hamsters exposed to short- and long-photoperiod. *International Journal of Andrology* **32** 486–497. (doi:10.1111/j.1365-2605.2008.00884.x)

Doan PL & Chute JP 2012 The vascular niche: home for normal and malignant hematopoietic stem cells. *Leukemia* **26** 54–62. (doi:10.1038/leu.2011.236)

Dobrinski I, Ogawa T, Avarbock MR & Brinster RL 1999 Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. *Molecular Reproduction and Development* **53** 142–148. (doi:10.1002/(SICI)1098-2795(199906)53:2<142::AID-MRD3>3.0.CO;2-O)

Dubois W & Callard GV 1993 Culture of intact Sertoli/germ cell units and isolated Sertoli cells from Squalus testis. II. Stimulatory effects of insulin and IGF-I on DNA synthesis in premeiotic stages. *Journal of Experimental Zoology* **267** 233–244. (doi:10.1002/jez.1402670217)

Dym M & Clermont Y 1970 Role of spermatogonia in the repair of the seminiferous epithelium following X-irradiation of the rat testis. *American Journal of Anatomy* **128** 265–282. (doi:10.1002/aja.1001280302)

Ebata KT, Zhang X & Nagano MC 2005 Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. *Molecular Reproduction and Development* **72** 171–181. (doi:10.1002/mrd.20324)

Gaytan F, Bellido C, Aguilar E & van Rooijen N 1994 Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. *Journal of Reproduction and Fertility* **102** 393–399. (doi:10.1530/jrf.0.1020393)

Giuili G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M & Cuzin F 2002 Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. *EMBO Reports* **3** 753–759. (doi:10.1093/embo-reports/kvf149)

Grisanti L, Falciatori I, Grasso M, Dovere L, Fera S, Muciaccia B, Fuso A, Berno V, Boitani C, Stefanini M *et al*. 2009 Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. *Stem Cells* **27** 3043–3052. (doi:10.1002/stem.206)

Griswold MD, Bishop PD, Kim KH, Ping R, Siiteri JE & Morales C 1989 Function of vitamin A in normal and synchronized seminiferous tubules. *Annals of the New York Academy of Sciences* **564** 154–172. (doi:10.1111/j.1749-6632.1989.tb25895.x)

Hasegawa K & Saga Y 2012 Retinoic acid signaling in Sertoli cells regulates organization of the blood-testis barrier through cyclical changes in gene expression. *Development* **139** 4347–4355. (doi:10.1242/dev.080119)

Hofmann MC, Braydich-Stolle L & Dym M 2005 Isolation of male germ-line stem cells; influence of GDNF. *Developmental Biology* **279** 114–124. (doi:10.1016/j.ydbio.2004.12.006)

Holdcraft RW & Braun RE 2004 Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. *Development* **131** 459–467. (doi:10.1242/dev.00957)

Huang YH, Chin CC, Ho HN, Chou CK, Shen CN, Kuo HC, Wu TJ, Wu YC, Hung YC, Chang CC *et al*. 2009 Pluripotency of mouse spermatogonial stem cells maintained by IGF-1-dependent pathway. *FASEB Journal* **23** 2076–2087. (doi:10.1096/fj.08-121939)

Huckins C 1971 The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anatomical Record* **169** 533–557. (doi:10.1002/ar.1091690306)

Hutson JC 1992 Development of cytoplasmic digitations between Leydig cells and testicular macrophages of the rat. *Cell and Tissue Research* **267** 385–389. (doi:10.1007/BF00302977)

Hutson JC 2006 Physiologic interactions between macrophages and Leydig cells. *Experimental Biology and Medicine* **231** 1–7.

Ikami K, Tokue M, Sugimoto R, Noda C, Kobayashi S, Hara K & Yoshida S 2015 Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. *Development* **142** 1582–1592. (doi:10.1242/dev.118695)

Jain S, Naughton CK, Yang M, Strickland A, Vij K, Encinas M, Golden J, Gupta A, Heuckeroth R, Johnson EM Jr & Milbrandt J 2004 Mice expressing a dominant-negative Ret mutation phenocopy human

Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. *Development* **131** 5503–5513. (doi:10.1242/dev.01421)

Jijiwa M, Kawai K, Fukihara J, Nakamura A, Hasegawa M, Suzuki C, Sato T, Enomoto A, Asai N, Murakumo Y *et al*. 2008 GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. *Genes to Cells* **13** 365–374. (doi:10.1111/j.1365-2443.2008.01171.x)

Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fassler R & Shinohara T 2008 Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. *Cell Stem Cell* **3** 533–542. (doi:10.1016/j.stem.2008.08.002)

Kanatsu-Shinohara M, Inoue K, Takashima S, Takehashi M, Ogonuki N, Morimoto H, Nagasawa T, Ogura A & Shinohara T 2012 Reconstitution of mouse spermatogonial stem cell niches in culture. *Cell Stem Cell* **11** 567–578. (doi:10.1016/j.stem.2012.06.011)

Kaucher AV, Oatley MJ & Oatley JM 2012 NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. *Biology of Reproduction* **86** 164, 1–11. (doi:10.1095/biolreprod.111.097386)

Kluin PM & de Rooij DG 1981 A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. *International Journal of Andrology* **4** 475–493. (doi:10.1111/j.1365-2605.1981.tb00732.x)

Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, Hofmann MC, Chan WY & Dym M 2009 The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. *Biology of Reproduction* **80** 707–717. (doi:10.1095/biolreprod.108.073809)

Kubota H & Brinster RL 2006 Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature Clinical Practice Endocrinology and Metabolism* **2** 99–108. (doi:10.1038/ncpendmet0098)

Kubota H, Avarbock MR & Brinster RL 2003 Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *PNAS* **100** 6487–6492. (doi:10.1073/pnas.0631767100)

Kubota H, Avarbock MR & Brinster RL 2004 Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *PNAS* **101** 16489–16494. (doi:10.1073/pnas.0407063101)

Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X & Rao CV 2001 Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Molecular Endocrinology* **15** 184–200. (doi:10.1210/mend.15.1.0586)

Li H, Palczewski K, Baehr W & Clagett-Dame M 2011 Vitamin A deficiency results in meiotic failure and accumulation of undifferentiated spermatogonia in prepubertal mouse testis. *Biology of Reproduction* **84** 336–341. (doi:10.1095/biolreprod.110.086157)

Lok D & de Rooij DG 1983 Spermatogonial multiplication in the Chinese hamster. III. Labelling indices of undifferentiated spermatogonia throughout the cycle of the seminiferous epithelium. *Cell and Tissue Kinetics* **16** 31–40.

Lok D, Jansen MT & de Rooij DG 1983 Spermatogonial multiplication in the Chinese hamster. II. Cell cycle properties of undifferentiated spermatogonia. *Cell and Tissue Kinetics* **16** 19–29.

Lu N, Sargent KM, Clopton DT, Pohlmeier WE, Brauer VM, McFee RM, Weber JS, Ferrara N, Silversides DW & Cupp AS 2013 Loss of vascular endothelial growth factor A (VEGFA) isoforms in the testes of male mice causes subfertility, reduces sperm numbers, and alters expression of genes that regulate undifferentiated spermatogonia. *Endocrinology* **154** 4790–4802. (doi:10.1210/en.2013-1363)

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M *et al*. 2000 Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287** 1489–1493. (doi:10.1126/science.287.5457.1489)

Meng J, Holdcraft RW, Shima JE, Griswold MD & Braun RE 2005 Androgens regulate the permeability of the blood-testis barrier. *PNAS* **102** 16696–16700. (doi:10.1073/pnas.0506084102)

Merlet J, Racine C, Moreau E, Moreno SG & Habert R 2007 Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. *PNAS* **104** 3615–3620. (doi:10.1073/pnas.0611421104)

Mitranond V, Sobhon P, Tosukhowong P & Chindaduangrat W 1979 Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. *Acta Anatomica* **103** 159–168. (doi:10.1159/000145007)

Moore A & Morris ID 1993 The involvement of insulin-like growth factor-I in local control of steroidogenesis and DNA synthesis of Leydig and non-Leydig cells in the rat testicular interstitium. *Journal of Endocrinology* **138** 107–114. (doi:10.1677/joe.0.1380107)

Nagano MC 2003 Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biology of Reproduction* **69** 701–707. (doi:10.1095/biolreprod.103.016352)

Nakagawa T, Nabeshima Y & Yoshida S 2007 Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Developmental Cell* **12** 195–206. (doi:10.1016/j.devcel.2007.01.002)

Nakagawa T, Sharma M, Nabeshima Y, Braun RE & Yoshida S 2010 Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science* **328** 62–67. (doi:10.1126/science.1182868)

Nalbandian A, Dettin L, Dym M & Ravindranath N 2003 Expression of vascular endothelial growth factor receptors during male germ cell differentiation in the mouse. *Biology of Reproduction* **69** 985–994. (doi:10.1095/biolreprod.102.013581)

Naughton CK, Jain S, Strickland AM, Gupta A & Milbrandt J 2006 Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. *Biology of Reproduction* **74** 314–321. (doi:10.1095/biolreprod.105.047365)

Nes WD, Lukyanenko YO, Jia ZH, Quideau S, Howald WN, Pratum TK, West RR & Hutson JC 2000 Identification of the lipophilic factor produced by macrophages that stimulates steroidogenesis. *Endocrinology* **141** 953–958. (doi:10.1210/en.141.3.953)

Nicholls PK, Harrison CA, Rainczuk KE, Wayne Vogl A & Stanton PG 2013 Retinoic acid promotes Sertoli cell differentiation and antagonises activin-induced proliferation. *Molecular and Cellular Endocrinology* **377** 33–43. (doi:10.1016/j.mce.2013.06.034)

Nowak DG, Woolard J, Amin EM, Konopatskaya O, Saleem MA, Churchill AJ, Ladomery MR, Harper SJ & Bates DO 2008 Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *Journal of Cell Science* **121** 3487–3495. (doi:10.1242/jcs.016410)

Oakberg EF 1971 Spermatogonial stem-cell renewal in the mouse. *Anatomical Record* **169** 515–531. (doi:10.1002/ar.1091690305)

Oatley JM, Avarbock MR, Telaranta AI, Fearon DT & Brinster RL 2006 Identifying genes important for spermatogonial stem cell self-renewal and survival. *PNAS* **103** 9524–9529. (doi:10.1073/pnas.0603332103)

Oatley JM, Avarbock MR & Brinster RL 2007 Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. *Journal of Biological Chemistry* **282** 25842–25851. (doi:10.1074/jbc.M703474200)

Oatley JM, Oatley MJ, Avarbock MR, Tobias JW & Brinster RL 2009 Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development* **136** 1191–1199. (doi:10.1242/dev.032243)

Oatley JM, Kaucher AV, Avarbock MR & Brinster RL 2010 Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. *Biology of Reproduction* **83** 427–433. (doi:10.1095/biolreprod.109.083352)

Oatley MJ, Kaucher AV, Racicot KE & Oatley JM 2011*a* Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. *Biology of Reproduction* **85** 347–356. (doi:10.1095/biolreprod.111.091330)

Oatley MJ, Racicot KE & Oatley JM 2011*b* Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. *Biology of Reproduction* **84** 639–645. (doi:10.1095/biolreprod.110.087320)

Ploemacher RE, van der Sluijs JP, Voerman JS & Brons NH 1989 An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. *Blood* **74** 2755–2763.

Raverdeau M, Gely-Pernot A, Feret B, Dennefeld C, Benoit G, Davidson I, Chambon P, Mark M & Ghyselinck NB 2012 Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *PNAS* **109** 16582–16587. (doi:10.1073/pnas.1214936109)

Rudolfsson SH, Wikstrom P, Jonsson A, Collin O & Bergh A 2004 Hormonal regulation and functional role of vascular endothelial growth factor a

in the rat testis. *Biology of Reproduction* **70** 340–347. (doi:10.1095/biolreprod.103.016816)

Sar M, Lubahn DB, French FS & Wilson EM 1990 Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* **127** 3180–3186. (doi:10.1210/endo-127-6-3180)

Sariola H & Saarma M 2003 Novel functions and signalling pathways for GDNF. *Journal of Cell Science* **116** 3855–3862. (doi:10.1242/jcs.00786)

Shinohara T, Orwig KE, Avarbock MR & Brinster RL 2001 Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *PNAS* **98** 6186–6191. (doi:10.1073/pnas.111158198)

Smith LB & Walker WH 2014 The regulation of spermatogenesis by androgens. *Seminars in Cell and Developmental Biology* **30** 2–13. (doi:10.1016/j.semcdb.2014.02.012)

Spinnler K, Kohn FM, Schwarzer U & Mayerhofer A 2010 Glial cell line-derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man. *Human Reproduction* **25** 2181–2187. (doi:10.1093/humrep/deq170)

Sun F, Xu Q, Zhao D & Degui Chen C 2015 Id4 marks spermatogonial stem cells in the mouse testis. *Scientific Reports* **5** 17594. (doi:10.1038/srep17594)

Tajima Y, Watanabe D, Koshimizu U, Matsuzawa T & Nishimune Y 1995 Insulin-like growth factor-I and transforming growth factor-alpha stimulate differentiation of type A spermatogonia in organ culture of adult mouse cryptorchid testes. *International Journal of Andrology* **18** 8–12. (doi:10.1111/j.1365-2605.1995.tb00928.x)

Takashima S, Kanatsu-Shinohara M, Tanaka T, Takehashi M, Morimoto H & Shinohara T 2011 Rac mediates mouse spermatogonial stem cell homing to germline niches by regulating transmigration through the blood-testis barrier. *Cell Stem Cell* **9** 463–475. (doi:10.1016/j.stem.2011.08.011)

Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A & Shinohara T 2015 Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. *Stem Cell Reports* **4** 489–502. (doi:10.1016/j.stemcr.2015.01.010)

Takeda H, Chodak G, Mutchnik S, Nakamoto T & Chang C 1990 Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor. *Journal of Endocrinology* **126** 17–25. (doi:10.1677/joe.0.1260017)

Tanaka T, Kanatsu-Shinohara M, Lei Z, Rao CV & Shinohara T 2016 The luteinizing hormone-testosterone pathway regulates mouse spermatogonial stem cell self-renewal by suppressing WNT5A expression in Sertoli cells. *Stem Cell Reports* **7** 279–291. (doi:10.1016/j.stemcr.2016.07.005)

Tegelenbosch RA & de Rooij DG 1993 A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutation Research* **290** 193–200. (doi:10.1016/0027-5107(93)90159-D)

Tolkunova EN, Malashicheva AB, Chikhirzhina EV, Kostyleva EI, Zeng W, Luo J, Dobrinskii I, Hierholzer A, Kemler R & Tomilin AN 2009 E-cadherin as a novel surface marker of spermatogonial stem cells. *Tsitologiia* **51** 212–218.

Tufro A, Teichman J, Banu N & Villegas G 2007 Crosstalk between VEGF-A/VEGFR2 and GDNF/RET signaling pathways. *Biochemical and Biophysical Research Communications* **358** 410–416. (doi:10.1016/j.bbrc.2007.04.146)

van Beek ME, Davids JA, van de Kant HJ & de Rooij DG 1984 Response to fission neutron irradiation of spermatogonial stem cells in different stages of the cycle of the seminiferous epithelium. *Radiation Research* **97** 556–569. (doi:10.2307/3576147)

van Keulen CJ & de Rooij DG 1975 Spermatogenetic clones developing from repopulating stem cells surviving a high dose of an alkylating agent. *Cell and Tissue Kinetics* **8** 543–551. (doi:10.1111/j.1365-2184.1975.tb01240.x)

Vernet N, Dennefeld C, Guillou F, Chambon P, Ghyselinck NB & Mark M 2006 Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. *EMBO Journal* **25** 5816–5825. (doi:10.1038/sj.emboj.7601447)

Walker WH 2010 Non-classical actions of testosterone and spermatogenesis. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365** 1557–1569. (doi:10.1098/rstb.2009.0258)

Wang RS, Yeh S, Chen LM, Lin HY, Zhang C, Ni J, Wu CC, di Sant'Agnese PA, deMesy-Bentley KL, Tzeng CR *et al*. 2006 Androgen receptor in sertoli cell is essential for germ cell nursery and junctional complex formation in mouse testes. *Endocrinology* **147** 5624–5633. (doi:10.1210/en.2006-0138)

Wang S, Wang X, Wu Y & Han C 2015 IGF-1R signaling is essential for the proliferation of cultured mouse spermatogonial stem cells by promoting the G2/M progression of the cell cycle. *Stem Cells and Development* **24** 471–483. (doi:10.1089/scd.2014.0376)

Wolbach SB & Howe PR 1925 Tissue Changes Following Deprivation of Fat-Soluble a Vitamin. *Journal of Experimental Medicine* **42** 753–777. (doi:10.1084/jem.42.6.753)

Yang Y, Feng Y, Feng X, Liao S, Wang X, Gan H, Wang L, Lin X & Han C 2016 BMP4 cooperates with retinoic acid to induce the expression of differentiation markers in cultured mouse spermatogonia. *Stem Cells International* **2016** 1–14. (doi:10.1155/2016/9536192)

Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X *et al*. 2002 Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *PNAS* **99** 13498–13503. (doi:10.1073/pnas.212474399)

Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T & Nabeshima Y 2006 The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development* **133** 1495–1505. (doi:10.1242/dev.02316)

Yoshida S, Sukeno M & Nabeshima Y 2007 A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science* **317** 1722–1726. (doi:10.1126/science.1144885)

Zhang FP, Poutanen M, Wilbertz J & Huhtaniemi I 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Molecular Endocrinology* **15** 172–183. (doi:10.1210/mend.15.1.0582)

Zhou J & Bondy C 1993 Anatomy of the insulin-like growth factor system in the human testis. *Fertility and Sterility* **60** 897–904. (doi:10.1016/S0015-0282(16)56294-3)

Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C *et al*. 2008*a* Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. *Biology of Reproduction* **78** 537–545. (doi:10.1095/biolreprod.107.064337)

Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA, Small C & Griswold MD 2008*b* Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. *Biology of Reproduction* **79** 35–42. (doi:10.1095/biolreprod.107.066795)

Zhu LJ, Hardy MP, Inigo IV, Huhtaniemi I, Bardin CW & Moo-Young AJ 2000 Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. *Biology of Reproduction* **63** 368–376. (doi:10.1095/biolreprod63.2.368)

Zirkin BR, Santulli R, Awoniyi CA & Ewing LL 1989 Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology **124** 3043–3049. (doi:10.1210/endo-124-6-3043)

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