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Kevin M. Sargent
University of Nebraska-Lincoln, kevin.sargent@huskers.unl.edu

Debra T. Rozell
University of Nebraska-Lincoln, dclopton1@unl.edu

Ningxia Lu
University of Nebraska-Lincoln

William E. Pohlmeier
University of Nebraska-Lincoln, wpohlmeier2@unl.edu

Andrea S. Cupp
University of Nebraska-Lincoln, acupp2@unl.edu

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VEGFA splicing: divergent isoforms regulate spermatogonial stem cell maintenance

Kevin M. Sargent[1], **Debra T. Clopton**[1], **Ningxia Lu**[1], **William E. Pohlmeier**[1], and **Andrea S. Cupp**[1]

Andrea S. Cupp: acupp2@unl.edu

[1]Department of Animal Science, University of Nebraska-Lincoln, A224i Animal Science Building, 3940 Fair Street, Lincoln NE 68583-0908, USA

Abstract

Despite being well-known for regulating angiogenesis in both normal and tumorigenic environments, vascular endothelial growth factor A (VEGFA) has been recently implicated in male fertility, namely in the maintenance of spermatogonial stem cells (SSC). The *VEGFA* gene can be spliced into multiple distinct isoforms that are either angiogenic or antiangiogenic in nature. Although studies have demonstrated the alternative splicing of VEGFA, including the divergent roles of the two isoform family types, many investigations do not differentiate between them. Data concerning VEGFA in the mammalian testis are limited, but the various angiogenic isoforms appear to promote seminiferous cord formation and to form a gradient across which cells may migrate. Treatment with either antiangiogenic isoforms of VEGFA or with inhibitors to angiogenic signaling impair these processes. Serendipitously, expression of KDR, the primary receptor for both types of VEGFA isoforms, was observed on male germ cells. These findings led to further investigation of the way that VEGFA elicits avascular functions within testes. Following treatment of donor perinatal male mice with either antiangiogenic VEGFA165b or angiogenic VEGFA164 isoforms, seminiferous tubules were less colonized following transplantation with cells from VEGFA165b-treated donors. Thus, VEGFA165b and possibly other antiangiogenic isoforms of VEGFA reduce SSC number either by promoting premature differentiation, inducing cell death, or by preventing SSC formation. Thus, angiogenic isoforms of VEGFA are hypothesized to promote SSC self-renewal, and the divergent isoforms are thought to balance one another to maintain SSC homeostasis in vivo.

Keywords

VEGFA164; VEGFA165b; Spermatogonia; Testis; Self-renewal

Correspondence to: Andrea S. Cupp, acupp2@unl.edu.

Compliance with ethical standards

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Introduction

Spermatogonial stem cell (SSC) biology is an emergent subject within reproduction with the aim of improving our understanding of male fertility. Whereas many factors have been identified that regulate SSC maintenance, little is known about the mechanisms by which they function. Recently, a complicated candidate has been implicated in the regulation of SSC maintenance: vascular endothelial growth factor A (VEGFA; Caires et al. 2012). VEGFA is a powerful mitogen that binds to a number of receptors and co-receptors to elicit its effects and was originally identified as having a role in regulating blood vessel development (Ferrara and Henzel 1989). VEGFA-targeting drugs are even available as anticancer therapeutics, since VEGFA expression is up-regulated in tumorigenesis, a highly angiogenic process (Ellis and Hicklin 2008; Ferrara et al. 2004; Goel and Mercurio 2013). VEGFA has been the topic of recent reviews with respect to cancer (de Brot et al. 2015; Eswarappa and Fox 2015; Kofler and Simons 2015; Papadimitriou et al. 2015; Ricci et al. 2015). What complicates the *Vegfa* gene and makes it an outstanding candidate in SSC biology, however, is that it can be spliced into divergent isoforms (Bates et al. 2002) with distinct physiological functions that are thought either to promote SSC self-renewal or to reduce SSC number (Caires et al. 2012). Thus, we plan here to review the literature with a particular focus on rodents and the ways that (1) the testis contributes to SSC maintenance (2) VEGFA is spliced and functions, and (3) alternative VEGFA splicing contributes to regulating the fate of SSCs.

How do cells of the testis contribute to maintenance of SSCs?

The mammalian testis is a repository of many different cell types (both somatic and germ) that function in tight collaboration to ensure male fertility. Coordinated effort between the various cells allows for the establishment of a specialized niche to foster germ cell development (Shinohara et al. 2001). This niche is home to the SSCs, a subpopulation of the undifferentiated type A spermatogonia. Type A spermatogonia are divided into three subtypes: A_{single} or A_s, A_{paired} or A_{pr} (2 cells connected by a cytoplasmic bridge), and $A_{aligned}$ or A_{al} (chains of 4, 8, or 16 cells; de Rooij 1998; Oakberg 1971). SSCs possess the ability either to self-renew to maintain a viable stem cell pool or eventually to migrate away from the SSC niche to differentiate into mature sperm (Fig. 1). Whether murine spermatogenesis is defined by asymmetric or symmetric stem cell division has been debated, although Nakagawa and others have demonstrated not only that SSCs divide into two daughters and follow the definition of symmetrical division, but also that some became KIT + (kit oncogene), suggesting a commitment to differentiation. Work from this laboratory has also demonstrated that transit-amplifying cells (A_{pr} and A_{al}) can detach from their cytoplasmic bridges and begin self-renewal when the stem cell pool has been compromised (Klein et al. 2010; Nakagawa et al. 2007, 2010). Thus, it is the balance of self-renewal and differentiation that is imperative for sustained male fertility resulting in continued spermatogenesis (Fig. 1). Chen and Liu (2015) have presented an elegant overview of factors contributing to SSC maintenance.

Primordial germ cells (PGCs) are the earliest precursor cells to SSCs and migrate from the hindgut to the gonad during mouse embryonic development (Mintz and Russell 1957;

Ozdzenski 1969). PGCs become gonocytes (also known as prospermatogonia or prespermatogonia) when they are enclosed within seminiferous cords formed by Sertoli and peritubular myoid cells, an event that occurs at embryonic day (E) 10.5 in mice and E11.5 in rats (de Rooij and Russell 2000). Gonocytes are mitotically arrested at E17 until proliferation resumes between postnatal day (P) 1.5 and P3 (Peters 1970). Between P4 and P5, gonocytes migrate from the center of seminiferous cords outward towards the basement membrane and are thought to transition to SSCs during this time in rodents (McGuinness and Orth 1992a; Orth et al. 2000). Transplantation of germ cells from either mice or rats at P4-P5 results in robust donor-derived spermatogenesis when microinjected into germ-cell-depleted recipients (McLean et al. 2003; Orwig et al. 2002). Proliferation and migration have been shown to occur simultaneously; however, there is no guarantee either will happen. These cells may also begin to differentiate or undergo apoptosis (Culty 2009; McGuinness and Orth 1992a, 1992b). The cells that do not migrate away from the seminiferous cords are thought to be especially likely to undergo apoptosis (Roosen-Runge and Leik 1968).

The largest component of seminiferous cords and tubules is the Sertoli cell, which, consequently, is also the major constituent of the SSC niche given that it houses and provides nourishment to male germ cells. However, structural support is also provided by peritubular myoid cells (the smooth-muscle-like cells) that surround seminiferous tubules and provide contractions to transport spermatozoa into the epididymides for further maturation and subsequent storage. Located within the interstitium, the area between the seminiferous tubules, are the androgen-producing Leydig cells, macrophages, and testicular vasculature. Interestingly, SSC clusters have been noted to be located in portions of the seminiferous tubules that border the interstitium rather than other tubules (Yoshida et al. 2007).

Whereas much is clearly known about PGCs and testis development, little is understood with regard to SSC biology; however, many factors have been identified that either promote SSC self-renewal or differentiation. Glial-cell-line-derived neurotrophic factor (GDNF) is considered to be the major effector of SSC self-renewal (Kubota et al. 2004; Meng et al. 2000; Naughton et al. 2006; Viglietto et al. 2000), together with fibroblast growth factor 2 (FGF2; Kanatsu-Shinohara et al. 2005). Each of these growth factors is produced by and released from Sertoli cells and bind their respective receptors on male germ cells. FGF2 (and other FGF ligands) appear to have their signal transduction mediated through FGFR1 (Takashima et al. 2015), whereas GDNF binds to a heterodimer receptor comprised of ret proto-oncogene (RET) and GDNF family receptor alpha 1 (GFRA1; Airaksinen and Saarma 2002; Suzuki et al. 2009). The binding of GDNF to its receptors initiates signaling through both the MEK (mitogen-activated protein kinase [MAPK]/extracellular signal-regulated kinase [ERK] kinase), ERK and phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (Akt) pathways, whereas FGF2 signaling is mediated via the MAPK cascade. Other effectors of SSC renewal are expressed by germ cells: B cell CLL/lymphoma 6, member B (BCL6B; Oatley et al. 2006), nanos homolog 2/3 (NANOS2/3; Sada et al. 2009), forkhead box O1 (FOXO1; Goertz et al. 2011), and ets variant 5 (ETV5; Chen et al. 2005). Chemokine (C-X-C motif) ligand 12 (CXCL12) and its receptor, chemokine (C-X-C motif) receptor 4 (CXCR4; Yang et al. 2013), have also been implicated in SSC self-renewal. Zinc finger and BTB domain containing 16 (ZBTB16, also known as PLZF) is a well-accepted

marker for all stages of undifferentiated spermatogonia, and some believe that it is also a proponent of self-renewal (Buaas et al. 2004). Lin-28 homolog A (LIN28) also marks all stages of undifferentiated spermatogonia (Zheng et al. 2009). Finally, two potential markers of the A_s subpopulation that may be considered SSCs have been discovered. Inhibitor of DNA binding 4 (ID4) promotes SSC self-renewal and was the first marker identified (Chan et al. 2014; Oatley et al. 2011). More recently, paired box 7 (PAX7)–positive spermatogonia represent a small subset that possess stem cell potential, are enriched in neonatal testes, and are long-lived (Aloisio et al. 2014). Although all these previously mentioned factors have been discovered in the mouse germ line, undifferentiated embryonic cell transcription factor 1 (UTF1) is expressed by a subset of undifferentiated spermatogonia that are maintained for a subsequent epithelial cycle rather than differentiating (van Bragt et al. 2008; Fig. 2).

As stated previously, many factors promote the differentiation of SSCs. The major indicator of differentiation, KIT, is detectable on germ cells, is expressed in the latest stages of undifferentiated spermatogonia, and is retained through the pachytene spermatocyte stage (Manova et al. 1990). Its ligand, stem cell factor (SCF or KITL) is secreted by Sertoli cells and maintains differentiated cells upon binding, in addition to being a major mediator of PGC migration to the embryonic gonad (de Rooij et al. 1999; Laird et al. 2011; Manova et al. 1993; Ohta et al. 2000, 2003). Neurogenin3 (NGN3 or NEUROG3) is a transcription factor expressed by most, if not all, subtypes of undifferentiated spermatogonia and is thus considered a marker of SSCs; however, the cells that differentiate and give rise to spermatogenesis have all been revealed to express it (Yoshida et al. 2004). Retinoic acid has also been demonstrated to induce the differentiation of spermatogonia and is thought to be produced by Sertoli cells (Bowles and Koopman 2007). Stimulated by retinoic acid gene 8 (STRA8) marks the entry into meiosis (Koubova et al. 2006). Testes of mice null for spermatogenesis and oogenesis specific basic helix-loop-helix 1/2 (SOHLH1/2) have KIT-positive cells that are prevented from entering the spermatocyte stage (Ballow et al. 2006; Toyoda et al. 2009). The pluripotency factor, sallike 4 (SALL4), is now considered to be a marker of undifferentiated spermatogonia because of its overlapping expression with PLZF: it appears to antagonize PLZF and allow for the induction of c-KIT (Gassei and Orwig 2013; Hobbs et al. 2012). Lastly, SRY (sex determining region Y)-box 3 (SOX3) has a similar expression pattern to those of SOHLH1/2 and NEUROG3 and, when knocked out, results in the depletion of meiotic male germ cells, although spermatogonia remain (Laronda and Jameson 2011; Raverot et al. 2005; Suzuki et al. 2012; Fig. 2).

Some factors have also been identified that are not produced by Sertoli or germ cells, whereas some even have functions other than self-renewal or differentiation. Thymus cell antigen 1, theta (THY1) has been shown to be a cell surface marker that enriches SSCs (Kubota et al. 2003). THY1-positive spermatogonia are enriched for colony stimulating factor 1 receptor (CSF1R), whose ligand, CSF1, is produced by Leydig cells and a small number of peritubular myoid cells (Oatley et al. 2009). Thus, interstitial cells secrete cytokines to promote self-renewal and maintain the SSC niche. Whereas SIN3A is a transcriptional regulator that is expressed by both Sertoli and germ cells within the testis, the Sertoli-cell-specific elimination of SIN3A in mice results in complete loss of spermatogenesis and failure of the SSC niche to be established (Payne et al. 2010). Finally, a recent study was the first to identify a factor that directly inhibits self-renewal, namely

ubiquitin ligase F-box and WD-40 domain protein 7 (FBXW7), and that targets c-Myc negatively to regulate self-renewal (Kanatsu-Shinohara et al. 2014). As stated previously, VEGFA has emerged as a novel regulator of SSC fate decisions.

How is VEGFA expression regulated?

VEGFA is a secreted glycoprotein growth factor that belongs to a family of VEGFs (with VEGFB, VEGFC, VEGFD, and placental growth factor [PGF]), of which VEGFA is the most studied (Carmeliet and Collen 1998; Ferrara and Henzel 1989). Since its discovery in 1989, over 55,500 papers appear in PubMed when searching for “VEGF”, a search for VEGF-A produces over 38,600 papers, and a search for “VEGFA” gives over 8500 hits. The *VEGFA* gene contains 8 exons and 7 introns with different splice variants of VEGFA characterized by the varied representation of exons 6 and 7 and thus altered lengths of the final translated product. *VEGFA* is located on chromosome 6 in humans (Vincenti et al. 1996), and four distinct isoforms of VEGFA have been identified (VEGFA206, VEGFA189, VEGFA165, and VEGFA121) named for the number of amino acids in the protein (Houck et al. 1991; Leung et al. 1989). This presence of multiple isoforms is thought to generate a chemoattractant gradient to stimulate cell migration within the testis (Bott et al. 2006). Larger isoforms such as VEGFA206 and VEGFA189 exhibit a lower mitogenic activity and stick close to the extracellular matrix because of multiple heparin-binding domains (Houck et al. 1991). VEGFA121 is the smallest, most soluble isoform, is the only one not containing a heparin-binding site, and is thus highly diffusible. The function of the most abundant isoform, VEGFA165, is considered intermediate, as it has one heparin-binding domain and is more diffusible than either VEGFA206 or VEGFA189. Rodent isoforms have been identified that are homologous to those found in humans but are shorter by one amino acid and are found on chromosome 17 (VEGFA205, VEGFA188, VEGFA164, and VEGFA120; De Gregorio et al. 1997; Ferrara et al. 1996) (Fig. 3).

Alternative splicing of the *VEGFA* gene generates either angiogenic or antiangiogenic isoforms thought either to promote vascular growth or to inhibit it, respectively. Sister antiangiogenic isoforms for angiogenic isoforms have also been discovered (VEGFA189b, VEGFA165b, VEGFA121b; Fig. 3). Rodent VEGFA antiangiogenic isoforms are named similarly. These antiangiogenic isoforms prevent cell migration and proliferation and inhibit the actions of angiogenic VEGFA isoforms. Since these inhibitory or “b” isoforms (VEGFAxxxb where “xxx” is the number of amino acids) are alternative splice variants of the same gene, different splice factors are phosphorylated in order to substitute the terminal six amino acids. Additionally, various other growth factors are known to regulate VEGFA splicing.

Transcription of the human *VEGFA* gene is induced by hypoxia (Blancher et al. 2000; Forsythe et al. 1996). Serine-arginine protein kinase 1 (SRPK1), however, is one of the molecules that phosphorylates serine/arginine-rich splicing factor 1 (SRSF1) to favor subsequent splicing at the 3′ proximal splice site in order to generate angiogenic isoforms of VEGFA (Amin et al. 2011; Mavrou et al. 2015). Upstream of these kinases that promote angiogenic splicing are other factors such as Wilms tumor 1 homolog (WT1), insulin-like growth factor 1 (IGF1), and transforming growth factor beta (TGFβ; Amin et al. 2011;

Nowak et al. 2008). Tumor necrosis factor alpha (TNFα) also increases total VEGFA protein while reducing expression of antiangiogenic isoforms, suggesting that it, too, favors the splicing of angiogenic VEGFA (Nowak et al. 2008; Fig. 3). In many homeostatic environments, other than the placenta, antiangiogenic isoforms are actually expressed more highly than the angiogenic isoforms (Bates et al. 2002; Bevan et al. 2008; Varey et al. 2008). Little information exists as to the factors that might generate antiangiogenic splice variants of VEGFA; however, the inhibition of SRPK1 and SRSF1 favor distal splice site targeting and the production of antiangiogenic VEGFA isoforms (Amin et al. 2011; Bates et al. 2002; Houck et al. 1991). One such factor known to inhibit SRPK1 and up-regulate antiangiogenic VEGFA165b is SRPIN340 (Amin et al. 2011; Oltean et al. 2012; Fig. 3). Interestingly, VEGFA is a target for some cancer treatments because of the demonstrated up-regulation of angiogenic isoforms of VEGFA and the concomitant reduction in antiangiogenic isoforms in renal and prostate carcinomas (Bates et al. 2002; Rennel et al. 2008). Thus, VEGFA requires tight regulation, and the two sister isoform types must be relatively balanced to maintain homeostasis within organs (Eremina et al. 2003). Otherwise, increased vasculature (higher expression of angiogenic VEGFA isoforms), as found in metastatic tissues, or decreased vasculature (higher expression of antiangiogenic VEGFA isoforms), as found in hypoxic situations, may result from dysregulation, all of which would be detrimental to the normal function of tissues (Bates et al. 2002; Konopatskaya et al. 2006; Rennel et al. 2008; Varey et al. 2008). More information regarding the splicing of *VEGFA* in humans, including novel splice variants not yet identified in rodents or with respect to SSC biology, has been recently reviewed but will not be included in here (Arcondeguy et al. 2013; Dehghanian et al. 2014).

How is VEGFA signaling mediated?

Both types of VEGFA family isoforms dimerize and signal through two tyrosine kinase receptors: FMS-like tyrosine kinase 1 (FLT1 or VEGFR1) and kinase insert domain receptor (KDR or VEGFR2; Ferrara and Henzel 1989). Whereas FLT1 appears to be important for cell migration amongst other processes and has a higher affinity for VEGFA than KDR (Kearney et al. 2004), FLT1 binding results in weak tyrosine phosphorylation that does not result in proliferation (Park et al. 1994; Waltenberger et al. 1994). Mice null for FLT1 die as early as E8.5–9 from vascular overgrowth suggesting that FLT1 acts as a sink to prevent too much VEGFA signal transduction through KDR, the receptor through which VEGFA elicits most of its biological activity (Ferrara et al. 2003; Fong et al. 1995; Roberts et al. 2004). Thus, FLT1 is thought to function as a dominant negative receptor to prevent too much signaling through KDR (Ferrara 2000).

Mice lacking KDR die between E8.5 and E9.5 from vascular defects (Shalaby et al. 1995). Although both angiogenic and antiangiogenic isoforms of VEGFA bind to KDR, they induce different conformational changes in the receptor. Additionally, antiangiogenic isoforms have been shown to result in different and less phosphorylation than angiogenic VEGFA (Cebe-Suarez et al. 2006; Kawamura et al. 2008; Woolard et al. 2004; Fig. 4).

VEGFA isoforms also bind to a class of membrane-bound co-receptors, the neuropilins. These membrane-bound co-receptors are normally known for their role in vasculogenesis and neuronal branching, since they also bind to semaphorins and plexins (Comeau et al.

1998; He and Tessier-Lavigne 1997). However, neuropilin-1 (NRP1) augments VEGFA signal transduction when it dimerizes and binds VEGFA to present the ligand to the KDR to stabilize the complex further at the plasma membrane (Cebe-Suarez et al. 2008; Soker et al. 1998; Yamada et al. 2001; Fig. 4). Both deletion and overexpression of NRP1 result in vascular defects and embryonic death in mice (Kawasaki et al. 1999; Kitsukawa et al. 1995). Human VEGFA121 lacks a heparin-binding site and, although it does bind NRP1, may not be able to be presented to KDR by NRP1, like other angiogenic isoforms (Pan et al. 2007). Heparin binding is thought to enhance the affinity of NRP1 for VEGFA angiogenic isoforms (Appleton et al. 2007). However, perhaps the most interesting aspect of NRP1 is its ability to bind only to angiogenic isoforms of VEGFA; this is thought to be because of its affinity for the 8a exon coding sequence (Cebe-Suarez et al. 2008; Harper and Bates 2008). Indeed, antiangiogenic isoforms of VEGFA lack any apparent NRP1-binding site (Cebe-Suarez et al. 2008). Thus, specific binding to NRP1 only occurs with VEGFA angiogenic isoforms; this is thought to enhance the signal transduction of these angiogenic isoforms and to amplify their effects on cells (Fig. 4).

How might VEGFA be more than an angiogenic factor within the testis?

Loss of even one allele of *Vegfa* in mice led to embryonic lethality between E11 and E12 (Ferrara et al. 1996). Expression of only *Vegfa164* resulted in normal mice, whereas the selective expression of either *Vegfa188* or *Vegfa120* caused either venous or arterial defects and dwarfism, presumably because of their molecular size and VEGFA164 functioning intermediately (Stalmans et al. 2002). VEGFA was initially investigated in the testis with regard to regulating male-specific vascular development following sexual differentiation. Whole testis mRNA abundance of *Vegfa188, Vegfa164* , and *Vegfa120* was detected at embryonic day E13 in the rat. These three isoforms plus *Vegfa205* were also expressed at E18 and P3 (Bott et al. 2006). *Kdr* was detected from E14 onward, whereas *Flt1* was not detected until E18, further supporting the role of KDR as the major mediator of VEGFA signaling and in testis-specific vascular development in this case (Bott et al. 2006). In the same study, the treatment of cultured rat testes with a VEGFA tyrosine kinase signal transduction inhibitor (VEGFA-TKI) reduced vascular density by over 50 % and inhibited seminiferous cord formation (Bott et al. 2006). Treatment of E13 rat testis organ cultures with VEGFA120, VEGFA164, or antiVEGFAxxxb (an antibody that neutralizes all antiangiogenic isoforms) increased vascular density by 48, 60, and 76 %, respectively (Baltes-Breitwisch et al. 2010). In another study, E11 testes from mice in which expression of the lacZ operon was driven by the *Kdr* promoter were also treated with VEGFA-TKI, which reduced β-galactosidase activity by 99 % after 3 days in culture (Bott et al. 2010). Prior to culture, positive cells were located mainly in the mesonephros and appeared to migrate into the testis, and KDR was observed in gonocytes and Sertoli cells at P4 and increasingly in meiotic germ cells from P20-P60 (Bott et al. 2010).

Where can VEGFA and its receptors be found within the testis?

VEGFA and its receptors are expressed by Sertoli, Leydig, and germ cells in patterns that coincide with germ cell recruitment and development in both rats and mice (Bott et al. 2006, 2010; Caires et al. 2012). Previously in the rat testis, a pan antibody for all VEGFA isoforms revealed expression primarily in Sertoli cells, around some germ cells, and in some

interstitial cells at E14, more highly in Sertoli cells surrounding germ cells at E16, and present in cords and more highly in germ than Sertoli cells at E19. By P0, VEGFA-positive staining was relegated to specific germ cells and then markedly increased within Sertoli cells by P5 in the rat testis (Bott et al. 2006). At similar time points, antiangiogenic VEGFA isoforms were expressed in specific germ cells and some interstitial cells but were largely absent from Sertoli cells (Baltes-Breitwisch et al. 2010).

VEGFA expression was examined around the time of SSC formation in mice. Positive staining for VEGFA angiogenic isoforms was detected mainly in Sertoli cells between P0 and P5 and to a lesser degree in germ cells, but this switched between P5 and P8 in mouse testes (Caires et al. 2012). Antiangiogenic isoforms, however, were expressed by subsets of gonocytes, undifferentiated spermatogonia, and Sertoli cells from P3-P5 and were expressed by meiotic germ cells at P20 (Caires et al. 2012).

KDR was expressed in Sertoli and germ cells at E16 and E17 (Bott et al. 2010). By P0, P4, and P5, KDR was also detectable in the germ cell cytoplasm and in some Sertoli cells (Bott et al. 2006, 2010). A series of experiments utilizing *Kdr-LacZ* mice was used to investigate KDR as a marker of vascularization within the developing testis; however, in addition to β-galactosidase-positive staining within the mesonephros and developing seminiferous cords, positive staining was clearly evident on germ cells at E17, P20, and P60 and on Leydig cells (Bott et al. 2010). These findings created a major turning point from which to begin investigations of VEGFA eliciting non-vascular effects.

A later study showed that KDR and FLT1 were expressed by gonocytes at P3, by spermatogonia at P5, by Sertoli cells, and consistently by Leydig cells (Caires et al. 2012). NRP1-positive staining was evident in gonocytes and Sertoli cells at P3, in undifferentiated spermatogonia at P5, and in spermatogonia from P6 to P20 (Caires et al. 2012). The same study investigated the activation of the receptors and co-receptor to determine whether their expression in these cell types at these time points meant that VEGFA was, indeed, a signaling molecule. Phosphorylation of FLT1 diminished after gonocyte migration to the basement membrane and was not detectable again until P20 in B spermatogonia, further implicating its role in cell migration and possibly survival (Caires et al. 2012). Both KDR and NRP1 were minimally activated, if at all, in Sertoli cells and gonocytes immediately after birth; however, activation coincided with gonocyte (P3-P8 for phospho-NRP1) and undifferentiated spermatogonia proliferation (P6–8 for phospho-KDR; Caires et al. 2012). Interestingly, activation of NRP1 in the same study was rarely detected at P14 and only in single undifferentiated spermatogonia, suggesting that NRP1 might play a distinct role in SSC maintenance. A tabular summary of the location of VEGFA ligands and receptors at various ages within the testis is included in this review (Table 1).

How do VEGFA isoforms potentially regulate SSC maintenance?

VEGFA has recently been implicated in the regulation of SSC fate. Treatment with either VEGFA164 or 165b has resulted in divergent effects on SSC maintenance. ROSA26 mice were treated daily from P3 to P5 with one of the two isoforms, and testes were collected at P8 for germ cell transplantation (Caires et al. 2012). The ROSA26 mice ubiquitously express the lacZ operon, and these donor germ cells are microinjected into the testes of

recipient mice (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). X-gal staining turns cells that are positive for β-galactosidase activity blue. Analysis of recipient testes showed a robust population of seminiferous tubules with blue cells following microinjection in mice treated with angiogenic VEGFA164 compared with a reduction in colonization following injection of VEGFA165b (Caires et al. 2012). These data demonstrated that VEGFA165b reduced SSC number; thus, we hypothesized that angiogenic isoforms promoted SSC proliferation (Fig. 5).

How might angiogenic VEGFA isoforms promote SSC self-renewal?

Following the study in which perinatal mice were treated with the various VEGFA isoforms, and after transplantation analyses had demonstrated that antiangiogenic isoforms reduced the number of SSCs, our group investigated the effect of the in vivo deletion of VEGFA on SSCs. Use of the Cre-lox system, *pDmrt1-Cre*, eliminated all *Vegfa* isoforms from both Sertoli and germ cells, and since exon 3 of *Vegfa* was floxed, this resulted in the successful knockout of both angiogenic and antiangiogenic isoforms (Lu et al. 2013). At 6 months of age, males lacking *Vegfa* were less fertile than controls and had alterations in mRNA abundance and immunostaining of factors known to regulate SSC maintenance. Whereas whole testes had increased *Ret*, *Sin3a*, and *Neurog3*, they had fewer PLZF-positive spermatogonia per tubule in addition to a 50% reduction in epididymal sperm. Positive cytoplasmic staining for phosphoFOXO1 was more evident in germ cells of mice lacking VEGFA compared with controls (Lu et al. 2013). As previously shown, FOXO1 is inactivated in the cytoplasm by phosphorylation and translocates to the nucleus as an activated unphosphorylated protein to promote SSC self-renewal (Goertz et al. 2011).

Earlier studies have shown that VEGFA164 signaling interacts with that of GDNF, and that VEGFA can phosphorylate RET (Tufro et al. 2007); moreover, GDNF is up-regulated by the addition of human VEGFA165, and KDR and RET are co-immunoprecipitated. Whereas these findings are related to the kidney, VEGFA165 induces the phosphorylation of tyrosine 1062 on the RET receptor, a phosphorylation site considered necessary for SSC self-renewal (Jain et al. 2004; Jijiwa et al. 2008) suggesting a possible link with VEGFA-GDNF-RET and SSCs. VEGFA transduces through multiple signaling pathways such as PI3K/AKT (Fujio and Walsh 1999) and MAPK (Takahashi et al. 2001) to promote cell migration, proliferation, and survival. The binding of both GDNF and FGF2 to their receptors initiates similar signal transduction (Baloh et al. 2000). GDNF and FGF2 have recently been demonstrated to promote SSC self-renewal at different rates, through different mechanisms, and can somewhat atone for one another as a result (Takashima et al. 2015). Perhaps Sertoli-cell-secreted angiogenic isoforms of VEGFA work similarly with these other growth factors (Fig. 6).

Recently, reactive oxygen species (ROS) have been shown to be effectors of SSC self-renewal (Morimoto et al. 2013). ROS and VEGFA are both up-regulated in hypoxic environments (Altavilla et al. 2012). In some tissue environments, the expression of VEGFA and its receptors has been increased by ROS (Fay et al. 2006; Kosmidou et al. 2001; Maraldi et al. 2010; Wang et al. 2011; Xia et al. 2007). Furthermore, VEGFA signaling through KDR is mediated, in part, by NADPH-induced oxidases (NOX), and NOX3 has just recently

been shown to be specifically involved in SSC self-renewal (Morimoto et al. 2015; Ushio-Fukai 2007). Finally, VEGFA signaling has been shown to be specifically mediated through NOX3 and NOX4 following enhancement by insulin, an inductor of angiogenic VEGFA splicing (Carnesecchi et al. 2006; Li et al. 2010; Meng et al. 2012). These findings represent possible links between VEGFA, ROS, and SSC self-renewal and thus provide further insight into the way that VEGFA modulates SSC fate.

How do antiangiogenic VEGFA isoforms reduce the number of SSCs?

We previously hypothesized that antiangiogenic isoforms of VEGFA reduce SSC number by promoting differentiation, by causing cell death, or perhaps even by inhibiting SSC formation. That being said, mammalian spermatogenesis is a highly productive process in that 256 spermatids can be generated from a single spermatogonium. However, the number actually produced is strikingly low, since 75 % of all spermatozoa are estimated to be lost to cell death (Print and Loveland 2000). In the rat, approximately half of all germ cells die at parturition (Roosen-Runge and Leik 1968), and apoptosis of male germ cells has been detected even while these cells are mitotically arrested (Coucouvanis et al. 1993). Autophagy has also been recently demonstrated in SSCs following the addition of a plasticizer in vitro (Liu et al. 2015). Treatment with angiogenic VEGFA164 has been demonstrated to promote germ cell survival, whereas inhibition of VEGFA signal transduction results in germ cell loss in cultured bovine testis explants (Caires et al. 2009). As a result, we hypothesize that antiangiogenic isoforms of VEGFA cause apoptosis in male germ cells.

Various studies have demonstrated that the pro-survival factor B cell leukemia/lymphoma 2 (BCL2) and proapoptotic factor BCL2-associated X protein (BAX) lie downstream of VEGFA signaling (Caires et al. 2009; Lu et al. 2013; Roberts et al. 2010; Street and Lenehan 2009; Suzuki et al. 2011). In our laboratory, 2– to 3-month-old *Kdr-LacZ* mice were injected with 1 μg recombinant antiangiogenic VEGFA165b. Significantly more TUNEL-positive apoptotic germ cells (spermatogonia) per seminiferous tubule were seen 24-h after VEGFA165b injection compared with controls suggesting that antiangiogenic VEGFA165b causes the apoptosis of male germ cells (Fig. 7).

How does the balance of VEGFA isoform types maintain SSC homeostasis?

VEGFA family isoforms may regulate the balance between a healthy SSC pool and progenitors that are more committed to differentiation. GFRA1-positive cells decrease as they transition from A_s to A_{al} (Nakagawa et al. 2010) suggesting the clonal cells lose stemness. Another study has demonstrated that the population of A_{al} cells that occur in chains of 8 or 16 contains very few to no cells that are GFRA1-positive, whereas all are ZBTB16 (PLZF)-positive (Grasso et al. 2012). Since RET is the receptor that heterodimerizes with GFRA1 to which GDNF binds, this might explain the opposite results observed with *Ret* mRNA and PLZF-positive staining following the loss of VEGFA isoforms. Perhaps the divergent VEGFA isoforms regulate the number of SSCs (angiogenic) to progenitor cells (antiangiogenic), similarly to the previously mentioned study in which FGF2 induces less self-renewal and favors cells that seem more likely to be progenitors as compared with GDNF (Takashima et al. 2015).

As mentioned earlier, NRP1 expression and activation appears to coincide with the gonocyte-to-SSC activation and are detectable in limited P20 spermatogonia (Caires et al. 2012). These data have led to the idea that NRP1 is a novel marker of SSCs. Since NRP1 has long been known to enhance the signaling of angiogenic VEGFA isoforms (Soker et al. 1998; Yamada et al. 2001), we suspect that NRP1 also contributes to the self-renewal of SSCs. NRP1 has been recently demonstrated to be important for the trafficking of KDR between endosomes (Lanahan et al. 2010, 2013) and the recycling of KDR to the plasma membrane in arteriogenesis (Ballmer-Hofer et al. 2011). If NRP1 is, indeed, relegated to the SSCs, its ability both to stabilize VEGFA-KDR binding and to recycle KDR suggests that it is a marker of SSC self-renewal. Perhaps NRP1 is the switch that shifts angiogenic to antiangiogenic activity and thus favors either self-renewal or death/differentiation of SSCs.

Concluding remarks

Aside from its canonical roles, VEGFA has also been demonstrated to play roles in reproductive organ development and fertility and, recently, in the maintenance of SSCs. Multiple studies have investigated the role of VEGFA in testis development, namely in the recruitment and formation of male-specific vascularization. Detection of the expression of KDR, the major receptor for all VEGFA isoforms, on male germ cells has revolutionized what was previously known, namely that VEGFA must have avascular effects in the testis. Further studies have subsequently demonstrated that the loss of VEGFA reduces fertility in male mice, and that divergent VEGFA isoforms even appear to regulate SSC maintenance in vivo. The induction of NRP1 expression in rodent spermatogonia also suggests that it plays an important role in angiogenic VEGFA signaling and SSC maintenance. Since treatment with antiangiogenic VEGFA165b results in less colonization of SSCs in recipient male mice, presumably because of SSC loss, we hypothesize that angiogenic isoforms of VEGFA and NRP1 promote SSC self-renewal, whereas antiangiogenic isoforms reduce SSC number by either promoting premature differentiation or death of SSCs. Some links occur between VEGFA164 and GDNF signaling and with ROS, and VEGFA165b appears to induce male germ cell apoptosis. Although the exact mechanisms are currently unknown, the divergent VEGFA isoforms differentially regulate SSC fate. Thus, rodent models involving treatment with or elimination of VEGFA or its receptors followed by transplantation experiments should provide valuable information as to the way that they influence SSC maintenance, and whether they are viable candidates for the improvement of declining male fertility.

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References

Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci. 2002; 3:383–394. [PubMed: 11988777]

Aloisio GM, Nakada Y, Saatcioglu HD, Pena CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I, Hamra FK, Castrillon DH. PAX7 expression defines germline stem cells in the adult testis. J Clin Invest. 2014; 124:3929–3944. [PubMed: 25133429]

Altavilla D, Romeo C, Squadrito F, Marini H, Morgia G, Antonuccio P, Minutoli L. Molecular pathways involved in the early and late damage induced by testis ischemia: evidence for a rational pharmacological modulation. Curr Med Chem. 2012; 19:1219–1224. [PubMed: 22300051]

Amin EM, Oltean S, Hua J, Gammons MV, Hamdollah-Zadeh M, Welsh GI, Cheung MK, Ni L, Kase S, Rennel ES, Symonds KE, Nowak DG, Royer-Pokora B, Saleem MA, Hagiwara M, Schumacher VA, Harper SJ, Hinton DR, Bates DO, Ladomery MR. WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. Cancer Cell. 2011; 20:768–780. [PubMed: 22172722]

Appleton BA, Wu P, Maloney J, Yin J, Liang WC, Stawicki S, Mortara K, Bowman KK, Elliott JM, Desmarais W, Bazan JF, Bagri A, Tessier-Lavigne M, Koch AW, Wu Y, Watts RJ, Wiesmann C. Structural studies of neuropilin/antibody complexes provide insights into semaphorin and VEGF binding. EMBO J. 2007; 26:4902–4912. [PubMed: 17989695]

Arcondeguy T, Lacazette E, Millevoi S, Prats H, Touriol C. VEGF-A mRNA processing, stability and translation: a paradigm for intricate regulation of gene expression at the post-transcriptional level. Nucleic Acids Res. 2013; 41:7997–8010. [PubMed: 23851566]

Ballmer-Hofer K, Andersson AE, Ratcliffe LE, Berger P. Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. Blood. 2011; 118:816–826. [PubMed: 21586748]

Ballow D, Meistrich ML, Matzuk M, Rajkovic A. Sohlh1 is essential for spermatogonial differentiation. Dev Biol. 2006; 294:161–167. [PubMed: 16564520]

Baloh RH, Enomoto H, Johnson EM Jr, Milbrandt J. The GDNF family ligands and receptors—implications for neural development. Curr Opin Neurobiol. 2000; 10:103–110. [PubMed: 10679429]

Baltes-Breitwisch MM, Artac RA, Bott RC, McFee RM, Kerl JG, Clopton DT, Cupp AS. Neutralization of vascular endothelial growth factor antiangiogenic isoforms or administration of proangiogenic isoforms stimulates vascular development in the rat testis. Reproduction. 2010; 140:319–329. [PubMed: 20457593]

Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer Res. 2002; 62:4123–4131. [PubMed: 12124351]

Bevan HS, Akker NM, van den, Qiu Y, Polman JA, Foster RR, Yem J, Nishikawa A, Satchell SC, Harper SJ, Gittenberger-de Groot AC, Bates DO. The alternatively spliced anti-angiogenic family of VEGF isoforms VEGFxxxb in human kidney development. Nephron Physiol. 2008; 110:57–67.

Blancher C, Moore JW, Talks KL, Houlbrook S, Harris AL. Relationship of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. Cancer Res. 2000; 60:7106–7113. [PubMed: 11156418]

Bott RC, McFee RM, Clopton DT, Toombs C, Cupp AS. Vascular endothelial growth factor and kinase domain region receptor are involved in both seminiferous cord formation and vascular development during testis morphogenesis in the rat. Biol Reprod. 2006; 75:56–67. [PubMed: 16672722]

Bott RC, Clopton DT, Fuller AM, McFee RM, Lu N, Cupp AS. KDR-LacZ-expressing cells are involved in ovarian and testis-specific vascular development, suggesting a role for VEGFA in the regulation of this vasculature. Cell Tissue Res. 2010; 342:117–130. [PubMed: 20848132]

Bowles J, Koopman P. Retinoic acid, meiosis and germ cell fate in mammals. Development. 2007; 134:3401–3411. [PubMed: 17715177]

van Bragt MP, Roepers-Gajadien HL, Korver CM, Bogerd J, Okuda A, Eggen BJ, de Rooij DG, van Pelt AM. Expression of the pluripotency marker UTF1 is restricted to a subpopulation of early A spermatogonia in rat testis. Reproduction. 2008; 136:33–40. [PubMed: 18390688]

Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A. 1994; 91:11303–11307. [PubMed: 7972054]

Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A. 1994; 91:11298–11302. [PubMed: 7972053]

de Brot S, Ntekim A, Cardenas R, James V, Allegrucci C, Heery DM, Bates DO, Odum N, Persson JL, Mongan NP. Regulation of vascular endothelial growth factor in prostate cancer. Endocr Relat Cancer. 2015; 22:R107–R123. [PubMed: 25870249]

Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet. 2004; 36:647–652. [PubMed: 15156142]

Caires KC, de Avila J, McLean DJ. Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis. Reproduction. 2009; 138:667–677. [PubMed: 19633133]

Caires KC, de Avila JM, Cupp AS, McLean DJ. VEGFA family isoforms regulate spermatogonial stem cell homeostasis in vivo. Endocrinology. 2012; 153:887–900. [PubMed: 22147017]

Carmeliet P, Collen D. Vascular development and disorders: molecular analysis and pathogenic insights. Kidney Int. 1998; 53:1519–1549. [PubMed: 9607184]

Carnesecchi S, Carpentier JL, Foti M, Szanto I. Insulin-induced vascular endothelial growth factor expression is mediated by the NADPH oxidase NOX3. Exp Cell Res. 2006; 312:3413–3424. [PubMed: 16949073]

Cebe-Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, Manlius C, Wood J, Ballmer-Hofer K. A VEGF-A splice variant defective for heparin sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. Cell Mol Life Sci. 2006; 63:2067–2077. [PubMed: 16909199]

Cebe-Suarez S, Grunewald FS, Jaussi R, Li X, Claesson-Welsh L, Spillmann D, Mercer AA, Prota AE, Ballmer-Hofer K. Orf virus VEGF-E NZ2 promotes paracellular NRP-1/VEGFR-2 coreceptor assembly via the peptide RPPR. FASEB J. 2008; 22:3078–3086. [PubMed: 18467594]

Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS, Oatley JM. Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev. 2014; 28:1351–1362. [PubMed: 24939937]

Chen C, Ouyang W, Grigura V, Zhou Q, Carnes K, Lim H, Zhao GQ, Arber S, Kurpios N, Murphy TL, Cheng AM, Hassell JA, Chandrashekar V, Hofmann MC, Hess RA, Murphy KM. ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature. 2005; 436:1030–1034. [PubMed: 16107850]

Chen SR, Liu YX. Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction. 2015; 149:R159–R167. [PubMed: 25504872]

Comeau MR, Johnson R, DuBose RF, Petersen M, Gearing P, VandenBos T, Park L, Farrah T, Buller RM, Cohen JI, Strockbine LD, Rauch C, Spriggs MK. A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity. 1998; 8:473–482. [PubMed: 9586637]

Coucouvanis EC, Sherwood SW, Carswell-Crumpton C, Spack EG, Jones PP. Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. Exp Cell Res. 1993; 209:238–247. [PubMed: 8262141]

Culty M. Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today Rev. 2009; 87:1–26.

De Gregorio L, Vincenti V, Breier G, Damert A, Dragani TA, Persico MG. Genetic mapping of the vascular endothelial growth factor (Vegf) gene to mouse chromosome 17. Mamm Genome. 1997; 8:451–452. [PubMed: 9166595]

Dehghanian F, Hojati Z, Kay M. New insights into VEGF-A alternative splicing: key regulatory switching in the pathological process. Avicenna J Med Biotechnol. 2014; 6:192–199. [PubMed: 25414781]

Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008; 8:579–591. [PubMed: 18596824]

Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest. 2003; 111:707–716. [PubMed: 12618525]

Eswarappa SM, Fox PL. Antiangiogenic VEGF-Ax: a new participant in tumor angiogenesis. Cancer Res. 2015; 75:2765–2769. [PubMed: 26122849]

Fay J, Varoga D, Wruck CJ, Kurz B, Goldring MB, Pufe T. Reactive oxygen species induce expression of vascular endothelial growth factor in chondrocytes and human articular cartilage explants. Arthritis Res Ther. 2006; 8:R189. [PubMed: 17187682]

Ferrara N. VEGF: an update on biological and therapeutic aspects. Curr Opin Biotechnol. 2000; 11:617–624. [PubMed: 11102799]

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun. 1989; 161:851–858. [PubMed: 2735925]

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 1996; 380:439–442. [PubMed: 8602242]

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003; 9:669–676. [PubMed: 12778165]

Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov. 2004; 3:391–400. [PubMed: 15136787]

Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 1995; 376:66–70. [PubMed: 7596436]

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 1996; 16:4604–4613. [PubMed: 8756616]

Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem. 1999; 274:16349–16354. [PubMed: 10347193]

Gassei K, Orwig KE. SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PLoS One. 2013; 8:e53976. [PubMed: 23326552]

Goel HL, Mercurio AM. VEGF targets the tumour cell. Nat Rev Cancer. 2013; 13:871–882. [PubMed: 24263190]

Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest. 2011; 121:3456–3466. [PubMed: 21865646]

Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, Vicini E. Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction. 2012; 143:325–332. [PubMed: 22143971]

Harper SJ, Bates DO. VEGF-A splicing: the key to anti-angiogenic therapeutics? Nat Rev Cancer. 2008; 8:880–887. [PubMed: 18923433]

He Z, Tessier-Lavigne M. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell. 1997; 90:739–751. [PubMed: 9288753]

Hobbs RM, Fagoonee S, Papa A, Webster K, Altruda F, Nishinakamura R, Chai L, Pandolfi PP. Functional antagonism between Sall4 and Plzf defines germline progenitors. Cell Stem Cell. 2012; 10:284–298. [PubMed: 22385656]

Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991; 5:1806–1814. [PubMed: 1791831]

Jain S, Naughton CK, Yang M, Strickland A, Vij K, Encinas M, Golden J, Gupta A, Heuckeroth R, Johnson EM Jr, Milbrandt J. Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. Development. 2004; 131:5503–5513. [PubMed: 15469971]

Jijiwa M, Kawai K, Fukihara J, Nakamura A, Hasegawa M, Suzuki C, Sato T, Enomoto A, Asai N, Murakumo Y, Takahashi M. GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. Genes Cells. 2008; 13:365–374. [PubMed: 18363967]

Kanatsu-Shinohara M, Ogonuki N, Iwano T, Lee J, Kazuki Y, Inoue K, Miki H, Takehashi M, Toyokuni S, Shinkai Y, Oshimura M, Ishino F, Ogura A, Shinohara T. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development. 2005; 132:4155–4163. [PubMed: 16107472]

Kanatsu-Shinohara M, Onoyama I, Nakayama KI, Shinohara T. Skp1-Cullin-F-box (SCF)-type ubiquitin ligase FBXW7 negatively regulates spermatogonial stem cell self-renewal. Proc Natl Acad Sci U S A. 2014; 111:8826–8831. [PubMed: 24879440]

Kawamura H, Li X, Harper SJ, Bates DO, Claesson-Welsh L. Vascular endothelial growth factor (VEGF)-A165b is a weak in vitro agonist for VEGF receptor-2 due to lack of coreceptor binding and deficient regulation of kinase activity. Cancer Res. 2008; 68:4683–4692. [PubMed: 18559514]

Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H. A requirement for neuropilin-1 in embryonic vessel formation. Development. 1999; 126:4895–4902. [PubMed: 10518505]

Kearney JB, Kappas NC, Ellerstrom C, DiPaola FW, Bautch VL. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. Blood. 2004; 103:4527–4535. [PubMed: 14982871]

Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H. Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development. 1995; 121:4309–4318. [PubMed: 8575331]

Klein AM, Nakagawa T, Ichikawa R, Yoshida S, Simons BD. Mouse germ line stem cells undergo rapid and stochastic turnover. Cell Stem Cell. 2010; 7:214–224. [PubMed: 20682447]

Kofler NM, Simons M. Angiogenesis versus arteriogenesis: neuropilin 1 modulation of VEGF signaling. F1000Prime Rep. 2015; 7:26. [PubMed: 25926977]

Konopatskaya O, Churchill AJ, Harper SJ, Bates DO, Gardiner TA. VEGF165b, an endogenous C-terminal splice variant of VEGF, inhibits retinal neovascularization in mice. Mol Vis. 2006; 12:626–632. [PubMed: 16735996]

Kosmidou I, Xagorari A, Roussos C, Papapetropoulos A. Reactive oxygen species stimulate VEGF production from C(2)C(12) skeletal myotubes through a PI3K/Akt pathway. Am J Physiol Lung Cell Mol Physiol. 2001; 280:L585–L592. [PubMed: 11237996]

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci U S A. 2006; 103:2474–2479. [PubMed: 16461896]

Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A. 2003; 100:6487–6492. [PubMed: 12738887]

Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A. 2004; 101:16489–16494. [PubMed: 15520394]

Laird DJ, Altshuler-Keylin S, Kissner MD, Zhou X, Anderson KV. Ror2 enhances polarity and directional migration of primordial germ cells. PLoS Genet. 2011; 7:e1002428. [PubMed: 22216013]

Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, Simons M. VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Dev Cell. 2010; 18:713–724. [PubMed: 20434959]

Lanahan A, Zhang X, Fantin A, Zhuang Z, Rivera-Molina F, Speichinger K, Prahst C, Zhang J, Wang Y, Davis G, Toomre D, Ruhrberg C, Simons M. The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. Dev Cell. 2013; 25:156–168. [PubMed: 23639442]

Laronda MM, Jameson JL. Sox3 functions in a cell-autonomous manner to regulate spermatogonial differentiation in mice. Endocrinology. 2011; 152:1606–1615. [PubMed: 21248142]

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989; 246:1306–1309. [PubMed: 2479986]

Li J, Wang JJ, Yu Q, Chen K, Mahadev K, Zhang SX. Inhibition of reactive oxygen species by Lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood-retinal barrier breakdown in db/db mice: role of NADPH oxidase 4. Diabetes. 2010; 59:1528–1538. [PubMed: 20332345]

Liu M, Wang J, Wei J, Xu L, Yu M, Liu X, Ruan W, Chen J. Triortho-cresyl phosphate induces autophagy of rat spermatogonial stem cells. Reproduction. 2015; 149:163–170. [PubMed: 25385720]

Lu N, Sargent KM, Clopton DT, Pohlmeier WE, Brauer VM, McFee RM, Weber JS, Ferrara N, Silversides DW, Cupp AS. 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. 2013; 154:4790–4802. [PubMed: 24169552]

Manova K, Nocka K, Besmer P, Bachvarova RF. Gonadal expression of c-kit encoded at the W locus of the mouse. Development. 1990; 110:1057–1069. [PubMed: 1712701]

Manova K, Huang EJ, Angeles M, De Leon V, Sanchez S, Pronovost SM, Besmer P, Bachvarova RF. The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. Dev Biol. 1993; 157:85–99. [PubMed: 7683286]

Maraldi T, Prata C, Caliceti C, Vieceli Dalla Sega F, Zambonin L, Fiorentini D, Hakim G. VEGF-induced ROS generation from NAD(P)H oxidases protects human leukemic cells from apoptosis. Int J Oncol. 2010; 36:1581–1589. [PubMed: 20428783]

Mavrou A, Brakspear K, Hamdollah-Zadeh M, Damodaran G, Babaei-Jadidi R, Oxley J, Gillatt DA, Ladomery MR, Harper SJ, Bates DO, Oltean S. Serine-arginine protein kinase 1 (SRPK1) inhibition as a potential novel targeted therapeutic strategy in prostate cancer. Oncogene. 2015; 34:4311–4319. [PubMed: 25381816]

McGuinness MP, Orth JM. Gonocytes of male rats resume migratory activity postnatally. Eur J Cell Biol. 1992a; 59:196–210. [PubMed: 1361432]

McGuinness MP, Orth JM. Reinitiation of gonocyte mitosis and movement of gonocytes to the basement membrane in testes of newborn rats in vivo and in vitro. Anat Rec. 1992b; 233:527–537. [PubMed: 1626712]

McLean DJ, Friel PJ, Johnston DS, Griswold MD. Characterization of spermatogonial stem cell maturation and differentiation in neonatal mice. Biol Reprod. 2003; 69:2085–2091. [PubMed: 12954735]

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science. 2000; 287:1489–1493. [PubMed: 10688798]

Meng D, Mei A, Liu J, Kang X, Shi X, Qian R, Chen S. NADPH oxidase 4 mediates insulin-stimulated HIF-1alpha and VEGF expression, and angiogenesis in vitro. PLoS One. 2012; 7:e48393. [PubMed: 23144758]

Mintz B, Russell ES. Gene-induced embryological modifications of primordial germ cells in the mouse. J Exp Zool. 1957; 134:207–237. [PubMed: 13428952]

Morimoto H, Iwata K, Ogonuki N, Inoue K, Atsuo O, Kanatsu-Shinohara M, Morimoto T, Yabe-Nishimura C, Shinohara T. ROS are required for mouse spermatogonial stem cell self-renewal. Cell Stem Cell. 2013; 12:774–786. [PubMed: 23746981]

Morimoto H, Kanatsu-Shinohara M, Shinohara T. ROS-generating oxidase Nox3 regulates the self-renewal of mouse spermatogonial stem cells. Biol Reprod. 2015; 92:147. [PubMed: 25947060]

Nakagawa T, Nabeshima Y, Yoshida S. Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell. 2007; 12:195–206. [PubMed: 17276338]

Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science. 2010; 328:62–67. [PubMed: 20299552]

Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod. 2006; 74:314–321. [PubMed: 16237148]

Nowak DG, Woolard J, Amin EM, Konopatskaya O, Saleem MA, Churchill AJ, Ladomery MR, Harper SJ, Bates DO. Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. J Cell Sci. 2008; 121:3487–3495. [PubMed: 18843117]

Oakberg EF. Spermatogonial stem-cell renewal in the mouse. Anat Rec. 1971; 169:515–531. [PubMed: 5550531]

Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A. 2006; 103:9524–9529. [PubMed: 16740658]

Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development. 2009; 136:1191–1199. [PubMed: 19270176]

Oatley MJ, Kaucher AV, Racicot KE, Oatley JM. Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. Biol Reprod. 2011; 85:347–356. [PubMed: 21543770]

Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development. 2000; 127:2125–2131. [PubMed: 10769236]

Ohta H, Tohda A, Nishimune Y. Proliferation and differentiation of spermatogonial stem cells in the w/wv mutant mouse testis. Biol Reprod. 2003; 69:1815–1821. [PubMed: 12890724]

Oltean S, Gammons M, Hulse R, Hamdollah-Zadeh M, Mavrou A, Donaldson L, Salmon AH, Harper SJ, Ladomery MR, Bates DO. SRPK1 inhibition in vivo: modulation of VEGF splicing and potential treatment for multiple diseases. Biochem Soc Trans. 2012; 40:831–835. [PubMed: 22817743]

Orth JM, Jester WF, Li LH, Laslett AL. Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. Curr Top Dev Biol. 2000; 50:103–124. [PubMed: 10948452]

Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. Proc Natl Acad Sci U S A. 2002; 99:11706–11711. [PubMed: 12185252]

Ozdzenski W. Fate of primordial germ cells in the transplanted hind gut of mouse embryos. J Embryol Exp Morphol. 1969; 22:505–510. [PubMed: 5360028]

Pan Q, Chathery Y, Wu Y, Rathore N, Tong RK, Peale F, Bagri A, Tessier-Lavigne M, Koch AW, Watts RJ. Neuropilin-1 binds to VEGF121 and regulates endothelial cell migration and sprouting. J Biol Chem. 2007; 282:24049–24056. [PubMed: 17575273]

Papadimitriou K, Rolfo C, Dewaele E, Van De Wiel M, Van den Brande J, Altintas S, Huizing M, Specenier P, Peeters M. Incorporating anti-VEGF pathway therapy as a continuum of care in metastatic colorectal cancer. Curr Treat Options in Oncol. 2015; 16:18.

Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem. 1994; 269:25646–25654. [PubMed: 7929268]

Payne CJ, Gallagher SJ, Foreman O, Dannenberg JH, Depinho RA, Braun RE. Sin3a is required by Sertoli cells to establish a niche for undifferentiated spermatogonia, germ cell tumors, and spermatid elongation. Stem Cells. 2010; 28:1424–1434. [PubMed: 20572009]

Peters H. Migration of gonocytes into the mammalian gonad and their differentiation. Philos Trans R Soc Lond B Biol Sci. 1970; 259:91–101. [PubMed: 4399071]

Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays. 2000; 22:423–430. [PubMed: 10797482]

Raverot G, Weiss J, Park SY, Hurley L, Jameson JL. Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol. 2005; 283:215–225. [PubMed: 15893302]

Rennel E, Waine E, Guan H, Schüler Y, Leenders W, Woolard J, Sugiono M, Gillatt D, Kleinerman E, Bates D, Harper S. The endogenous anti-angiogenic VEGF isoform, VEGF165b inhibits human tumour growth in mice. Br J Cancer. 2008; 98:1250–1257. [PubMed: 18349828]

Ricci V, Ronzoni M, Fabozzi T. Aflibercept a new target therapy in cancer treatment: a review. Crit Rev Oncol Hematol. 2015 (in press).

Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. Am J Pathol. 2004; 164:1531–1535. [PubMed: 15111299]

Roberts OL, Holmes K, Muller J, Cross DA, Cross MJ. ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells. J Cell Sci. 2010; 123:3189–3200. [PubMed: 20736307]

de Rooij DG. Stem cells in the testis. Int J Exp Pathol. 1998; 79:67–80. [PubMed: 9709376]

de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl. 2000; 21:776–798. [PubMed: 11105904]

de Rooij DG, Okabe M, Nishimune Y. Arrest of spermatogonial differentiation in jsd/jsd, Sl17H/Sl17H, and cryptorchid mice. Biol Reprod. 1999; 61:842–847. [PubMed: 10456866]

Roosen-Runge EC, Leik J. Gonocyte degeneration in the postnatal male rat. Am J Anat. 1968; 122:275–299. [PubMed: 5665153]

Sada A, Suzuki A, Suzuki H, Saga Y. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science. 2009; 325:1394–1398. [PubMed: 19745153]

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995; 376:62–66. [PubMed: 7596435]

Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A. 2001; 98:6186–6191. [PubMed: 11371640]

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell. 1998; 92:735–745. [PubMed: 9529250]

Stalmans I, Ng YS, Rohan R, Fruttiger M, Bouche A, Yuce A, Fujisawa H, Hermans B, Shani M, Jansen S, Hicklin D, Anderson DJ, Gardiner T, Hammes HP, Moons L, Dewerchin M, Collen D, Carmeliet P, D'Amore PA. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest. 2002; 109:327–336. [PubMed: 11827992]

Street J, Lenehan B. Vascular endothelial growth factor regulates osteoblast survival—evidence for an autocrine feedback mechanism. J Orthop Surg Res. 2009; 4:19. [PubMed: 19527527]

Suzuki H, Sada A, Yoshida S, Saga Y. The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Dev Biol. 2009; 336:222–231. [PubMed: 19818747]

Suzuki H, Ahn HW, Chu T, Bowden W, Gassei K, Orwig K, Rajkovic A. SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. Dev Biol. 2012; 361:301–312. [PubMed: 22056784]

Suzuki M, Ozawa Y, Kubota S, Hirasawa M, Miyake S, Noda K, Tsubota K, Kadonosono K, Ishida S. Neuroprotective response after photodynamic therapy: role of vascular endothelial growth factor. J Neuroinflammation. 2011; 8:176. [PubMed: 22171708]

Takahashi T, Yamaguchi S, Chida K, Shibuya M. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J. 2001; 20:2768–2778. [PubMed: 11387210]

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

Toyoda S, Miyazaki T, Miyazaki S, Yoshimura T, Yamamoto M, Tashiro F, Yamato E, Miyazaki J. Sohlh2 affects differentiation of KIT positive oocytes and spermatogonia. Dev Biol. 2009; 325:238–248. [PubMed: 19014927]

Tufro A, Teichman J, Banu N, Villegas G. Crosstalk between VEGF-A/VEGFR2 and GDNF/RET signaling pathways. Biochem Biophys Res Commun. 2007; 358:410–416. [PubMed: 17490619]

Ushio-Fukai M. VEGF signaling through NADPH oxidase-derived ROS. Antioxid Redox Signal. 2007; 9:731–739. [PubMed: 17511588]

Varey AH, Rennel ES, Qiu Y, Bevan HS, Perrin RM, Raffy S, Dixon AR, Paraskeva C, Zaccheo O, Hassan AB, Harper SJ, Bates DO. VEGF165b, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. Br J Cancer. 2008; 98:1366–1379. [PubMed: 18349829]

Viglietto G, Dolci S, Bruni P, Baldassarre G, Chiariotti L, Melillo RM, Salvatore G, Chiappetta G, Sferratore F, Fusco A, Santoro M. Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of Ret-expressing spermatogonia. Int J Oncol. 2000; 16:689–694. [PubMed: 10717236]

Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation. 1996; 93:1493–1495. [PubMed: 8608615]

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 1994; 269:26988–26995. [PubMed: 7929439]

Wang Y, Zang QS, Liu Z, Wu Q, Maass D, Dulan G, Shaul PW, Melito L, Frantz DE, Kilgore JA, Williams NS, Terada LS, Nwariaku FE. Regulation of VEGF-induced endothelial cell migration by mitochondrial reactive oxygen species. Am J Physiol Cell Physiol. 2011; 301:C695–C704. [PubMed: 21653897]

Woolard J, Wang WY, Bevan HS, Qui Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Muchens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res. 2004; 64:7822–7835. [PubMed: 15520188]

Xia C, Meng Q, Liu LZ, Rojanasakul Y, Wang XR, Jiang BH. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. Cancer Res. 2007; 67:10823–10830. [PubMed: 18006827]

Yamada Y, Takakura N, Yasue H, Ogawa H, Fujisawa H, Suda T. Exogenous clustered neuropilin 1 enhances vasculogenesis and angiogenesis. Blood. 2001; 97:1671–1678. [PubMed: 11238106]

Yang QE, Kim D, Kaucher A, Oatley MJ, Oatley JM. CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. J Cell Sci. 2013; 126:1009–1020. [PubMed: 23239029]

Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T, Nabeshima Y. Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol. 2004; 269:447–458. [PubMed: 15110712]

Yoshida S, Sukeno M, Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science. 2007; 317:1722–1726. [PubMed: 17823316]

Zheng K, Wu X, Kaestner KH, Wang PJ. The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol. 2009; 9:38. [PubMed: 19563657]

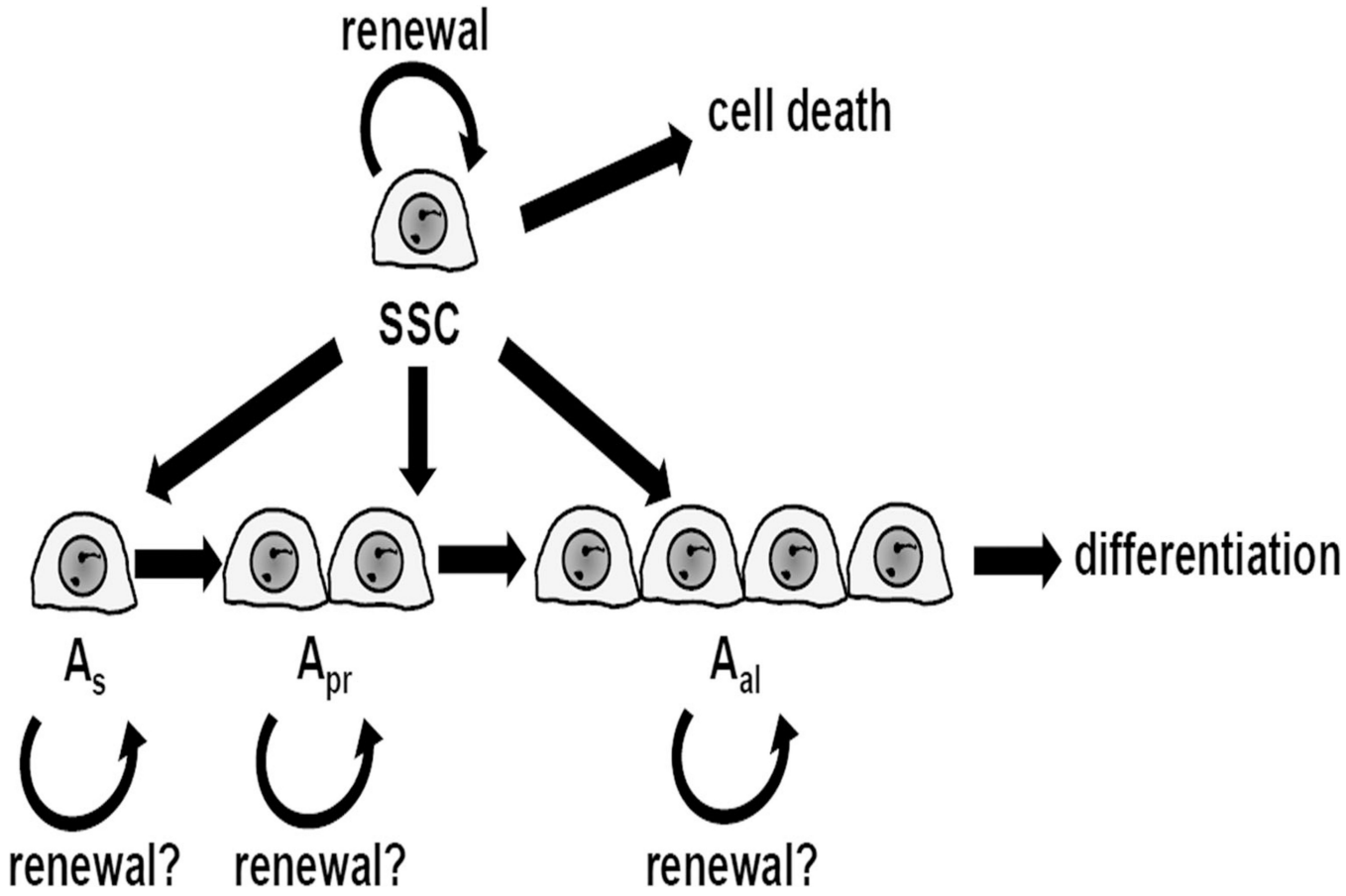

Fig. 1.

Model of spermatogonial stem cell (*SSC*) homeostasis. SSCs are considered a subpopulation of the undifferentiated A spermatogonia and may divide into other SSCs, divide into progenitor cells more committed to differentiation, or undergo cell death. The various stages of A undifferentiated spermatogonia, even when chained, are also thought to separate and resume self-renewal capabilities to maintain a viable SSC pool (A_s [A_{single}] type A spermatogonia, A_{pr} [A_{paired}] type A spermatogonia, A_{al} [$A_{aligned}$] type A spermatogonia)

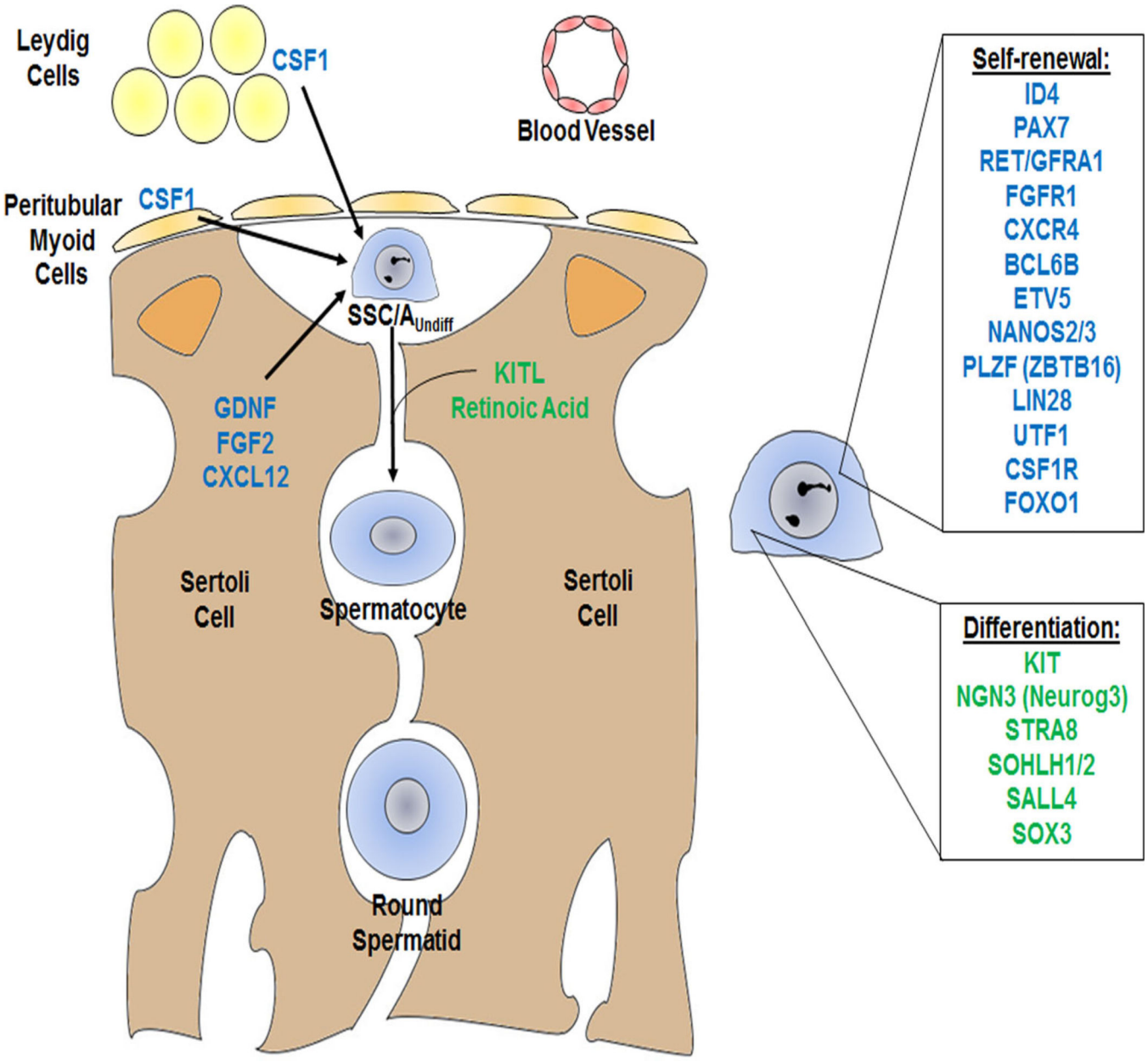

Fig. 2.

Spermatogonial stem cell (*SSC*) niche comprised of Sertoli cells with contributions from blood vessels, peritubular myoid cells, and Leydig cells. Various factors that contribute either to self-renewal or to differentiation are depicted in the cells from which they are produced. The large inset SSC (*right*) shows the pro-renewal or pro-differentiation factors that are expressed by the germ cells

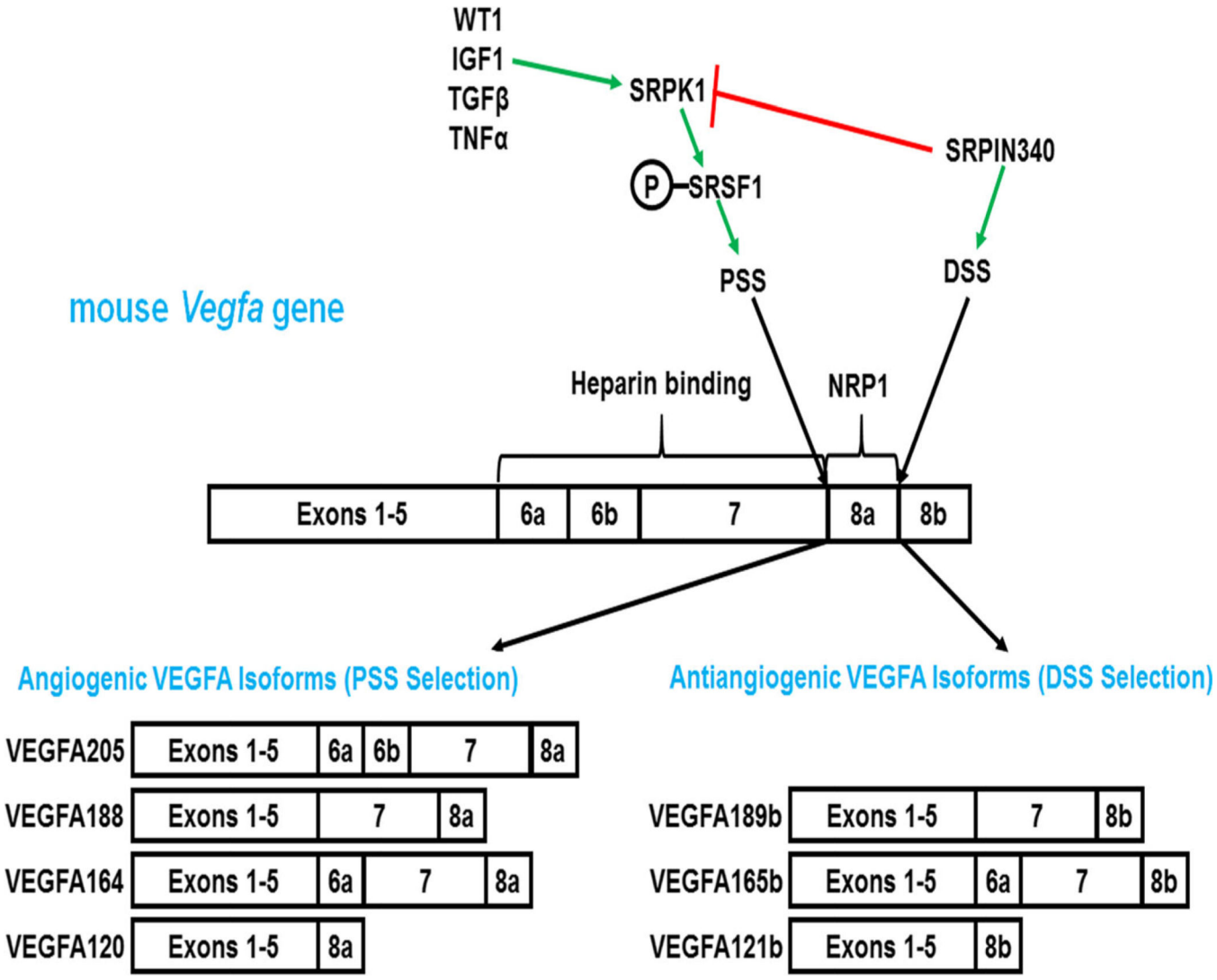

Fig. 3.

Depiction of rodent VEGFA isoforms. Angiogenic isoforms include VEGFA205, 188, 164, and 120, whereas VEGFA189b, 165b, and 121b are antiangiogenic. Various growth factors activate serine-arginine protein kinase 1 (*SRPK1*), which phosphorylates serine/arginine-rich splicing factor 1 (*SRSF1*) to target the proximal splice site (*PSS*) of the *Vegfa* gene and generate angiogenic VEGFA isoforms. Known, biologically active, rodent, angiogenic VEGFA isoforms are 205, 188, 164, and 120. Inhibition of SRPK1 by specific inhibitors such as SRPIN340 favors distal splice site (*DSS*) selection and the generation of antiangiogenic isoforms. Antiangiogenic isoforms contain an 8b exon that is substituted for an 8a exon in angiogenic VEGFA isoforms. VEGFA206189b, 165b, and 121b are the most well-represented rodent antiangiogenic isoforms in the literature. Exons 6 and 7 encode the heparin-binding sites, whereas the neuropilin-1 (*NRP1*)-binding site is encoded by translation of the 8a exon; this is the reason that it cannot bind antiangiogenic VEGFA isoforms

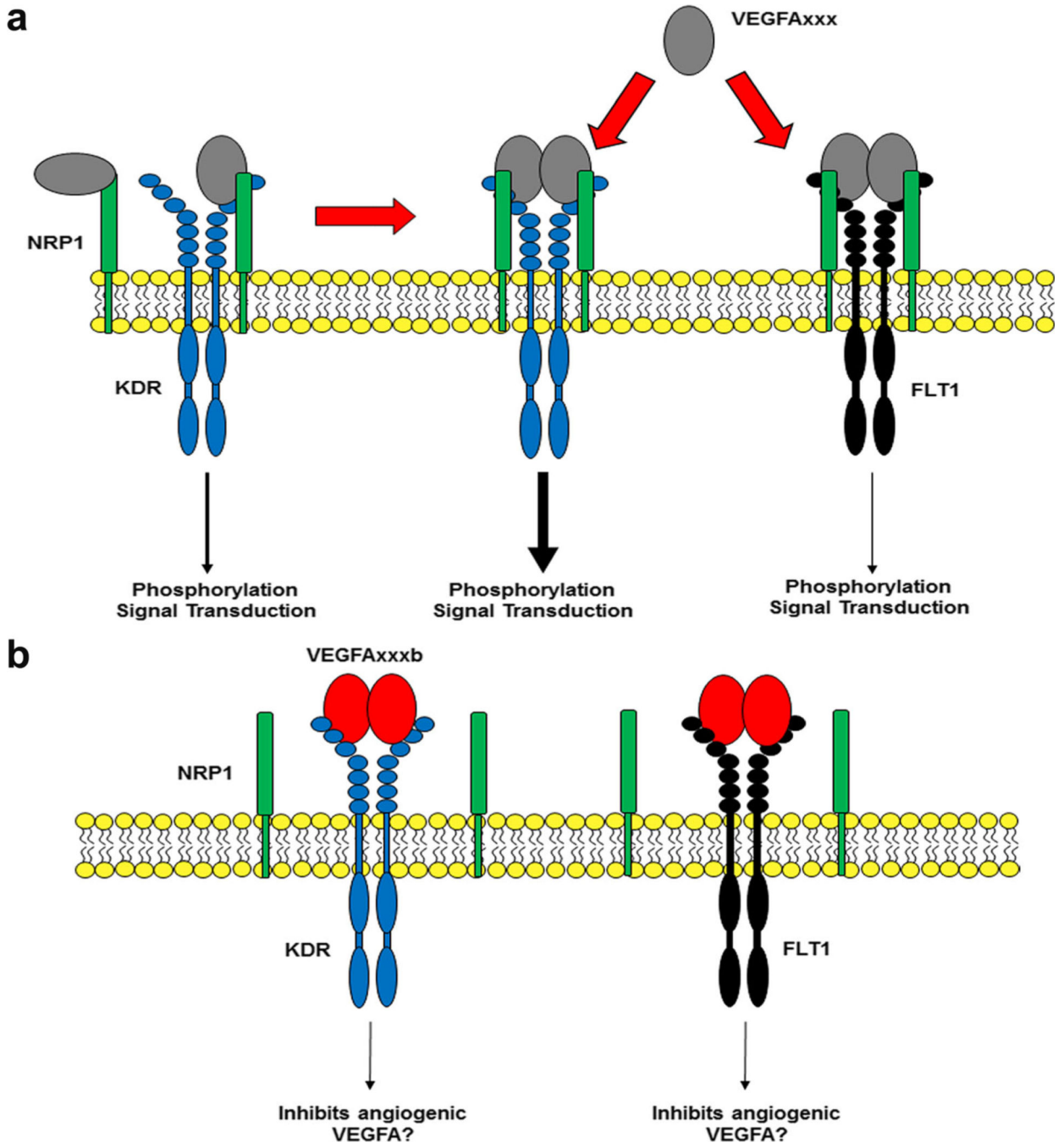

Fig. 4.

VEGFA signals through two tyrosine kinase receptors. Signaling of angiogenic isoforms of VEGFA is augmented by a membrane-bound co-receptor, NRP1, which either stabilizes ligand-receptor binding or presents the ligand to the kinase insert domain receptor (**a**, *KDR*, *left*). NRP1 also associates with VEGFA homodimers that bind to FMS-like tyrosine kinase 1 (**a**, *FLT1*, *right*). Antiangiogenic isoforms of VEGFA, however, are unable to bind to NRP1 but still bind either KDR (**b**, *left*) or FLT1 (**b**, *right*)

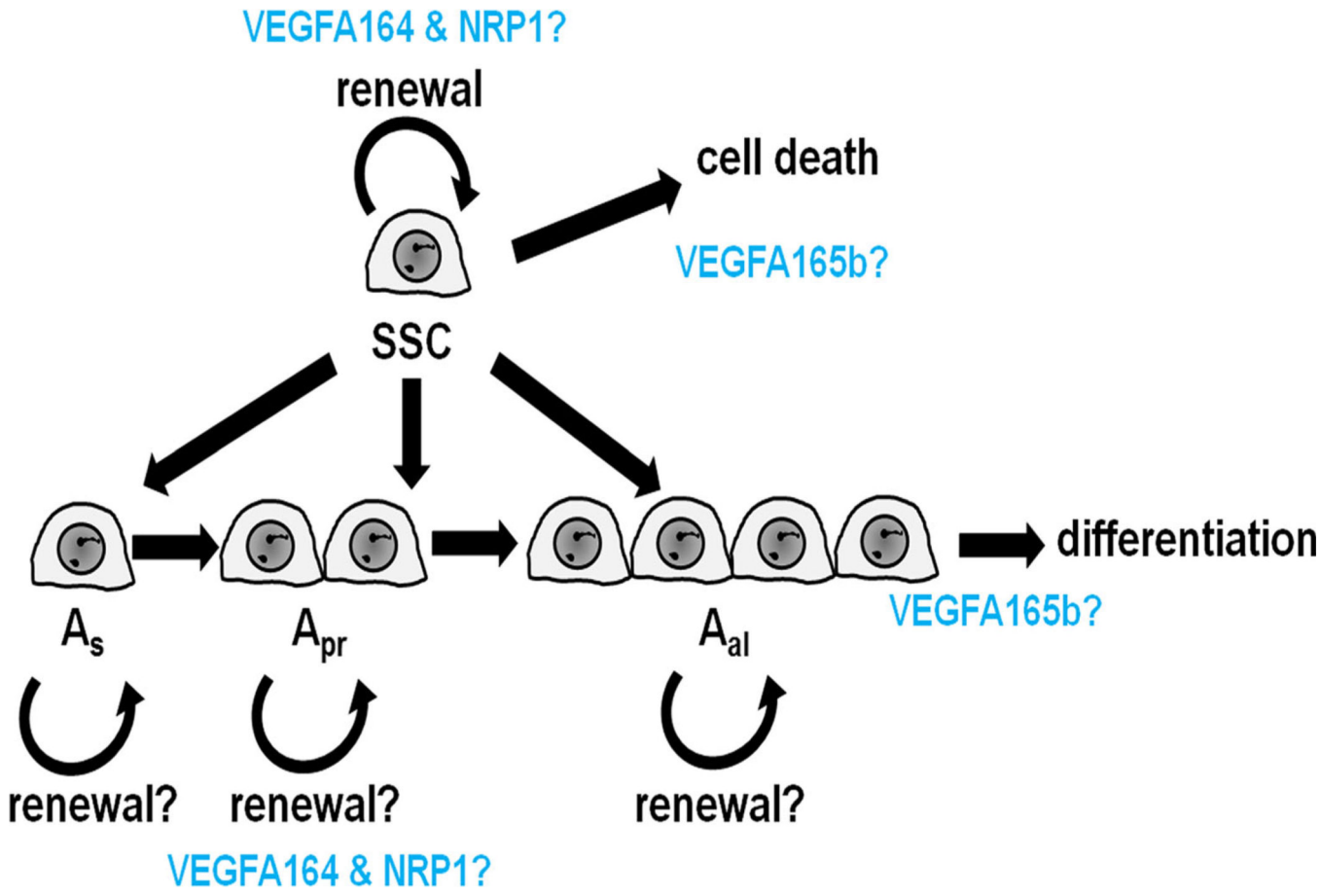

Fig. 5.

Model of SSC homeostasis with VEGFA isoforms and NRP1 added. We propose that angiogenic isoforms such as VEGFA164 and NRP1 promote the renewal of SSCs or undifferentiated spermatogonia, whereas antiangiogenic isoforms such as VEGFA165b reduce SSC number through cell death or by promoting differentiation prematurely

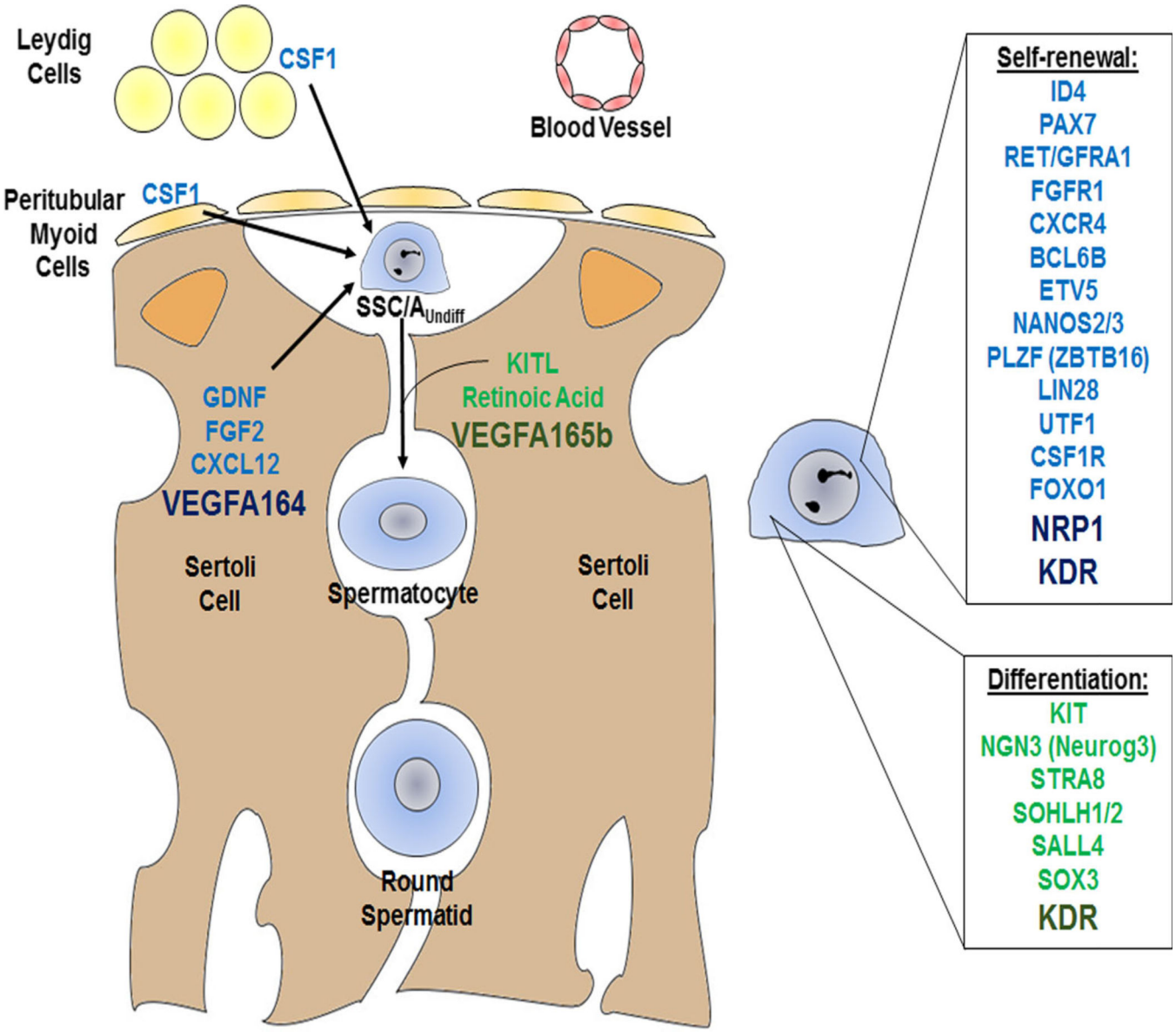

Fig. 6.

SSC niche with VEGFA isoforms added. We presume that angiogenic isoforms of VEGFA promote self-renewal of SSCs, whereas antiangiogenic isoforms of VEGFA promote their differentiation (or death), with these isoforms being predominantly secreted by the Sertoli cell. Furthermore, KDR and NRP1 are located on the germ cells themselves

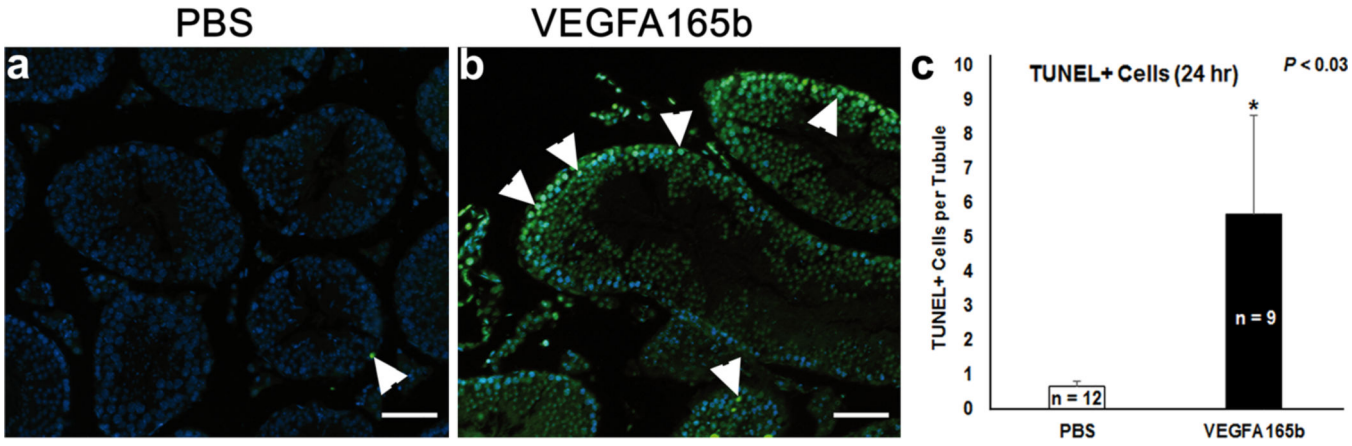

Fig. 7.

VEGFA165b increases TUNEL-positive staining in male germ cells. Micrographs are representative 100× images of testes from a phosphate-buffered saline (*PBS*, control)-treated mouse (**a**) and a VEGFA165b (1 μg)-treated mouse (**b**). Apoptotic spermatogonia fluoresced green (*white arrows*). The number of TUNEL-positive germ cells per tubule was counted for each treatment (**c**). Data were considered significant when $P<0.05$ according to Dunnett's test in JMP statistical discovery software from SAS

Table 1

Location of VEGFA isoform and receptor protein in the rodent testis

Age	VEGFA164	VEGFA165b	KDR	FLT1	NRP1
E14	Sertoli, germ, interstitium	Germ cells	Sertoli, Leydig		
E16	Sertoli surrounding germ	Sertoli surrounding germ	Sertoli, Leydig		
E17			Germ		
E19			Germ, Sertoli		
P0	Sertoli, germ	Sertoli, germ	Germ		
P3	Sertoli, germ	Sertoli, gonocytes, spermatogonia	Gonocytes	Gonocytes, Sertoli, Leydig	Gonocytes, Sertoli, Leydig
P5	Sertoli, germ		Spermatogonia	Spermatogonia, Sertoli, Leydig	Spermatogonia, Sertoli
P8	Sertoli, germ				Spermatogonia
P14					Spermatogonia
P20		Meiotic germ cells	Germ		Spermatogonia
P60			Sertoli, germ		