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Treatment with or Elimination of Vascular Endothelial Growth Factor A (VEGFA) Results in Altered mRNA Abundance of Undifferentiated Spermatogonia Niche, Renewal and Differentiation Factors

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Treatment with or Elimination of Vascular Endothelial Growth Factor A (VEGFA) Results in Altered mRNA Abundance of Undifferentiated Spermatogonia Niche, Renewal and Differentiation Factors

By

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Vascular Endothelial Growth Factor A (VEGFA) and its receptors are important for vascular development in the testis. Roles of VEGFA have been proposed in undifferentiated spermatogonia where proangiogenic isoforms are suggested to promote their self-renewal while antiangiogenic isoforms stimulate their differentiation. Thus, we treated male rats in vivo (perinatally with IP injections from P0-P2) with different VEGFA isoforms or an antibody to VEGFAxxxb (antiangiogenic isoforms) to determine if this would affect testis morphogenesis, expression of genes regulating the spermatogonial stem cell (SSC) niche and genes that regulate cell survival. Testis morphogenesis with VEGFA164 (1µg) isoform treatment increased cord area, reduced interstitium and tended to increase germ cell numbers while VEGFA165b (0.5 µg) decreased cord area and increased interstitium. The antiVEGFAxxxb treatment reduced expression of mRNA for Nanos2 and Ret- two genes important in SSC renewal. Furthermore, mRNA for Bax, a pro-apoptotic gene, was reduced, and the ratio of Bcl2:Bax tended to be increased. Serum testosterone tended to be increased with the antiVEGFAxxxb treatment while it was reduced with perinatal VEGFA165b (0.5 µg) treatment. A second study was performed with Amhr2-cre;Nrp-1+/− mice where Nrp-1,
co-receptor that binds proangiogenic isoforms of VEGFA, was knocked out in Sertoli cells. Mice at 78.6 days had reduced Bcl2 (pro-survival), Sin3a (niche establishment), Gdnf (SSC renewal), Neurog3 (differentiation) with a tendency for Ret and Kitl (survival and differentiation) to be reduced. Both the mRNA and the protein for PLZF (renewal) were elevated in knockout testes. Knockout males were mated to control females and allowed to breed until cessation. Compared to control matings, the knockout male matings to controls resulted in fewer pups per litter, a tendency for increased days between parturitions and fewer pups surviving to weaning. These data further confirm the balance of VEGFA isoforms is necessary for appropriate expression of genes regulating the SSC niche, and cell survival and they are also critical for male fertility.
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Chapter 1

Literature Review

Introduction

Infertility is an issue faced by many couples (10-15%), with half of cases attributable to men (Ferlin et al., 2011), where approximately 8% of men at reproductive age (15-49 years old) experience infertility (Esteves et al., 2011; Sonfield, 2002). Laboratory-based technologies have demonstrated that thousands of gene product pathways and signaling cascades from throughout the body crosstalk to result in reproductive viability, and even seemingly miniscule disruptions in these processes may result in infertility (Matzuk and Lamb, 2008). Ferlin and others (2011) also state that male infertility is often a result of highly diminished sperm numbers to completely aberrant spermatogenesis. Testicular dysgenesis syndrome (TDS) is hypothesized to be a composite disorder comprised of poor semen quality, undescended testes, testicular cancers and hypospadias (Funke et al., 2010). These four reproductive detriments- low semen quality, undescended testes, testicular cancers and hypospadias- are risk factors for each other. Causes for TDS have been linked to both genetic and environmental factors (Jorgensen et al., 2010), and most of the TDS is thought to be of fetal origin and the result of alterations in environmental effectors (Jorgensen et al., 2011). However, there is a possibility that low sperm counts are also associated with reductions or alterations in undifferentiated spermatogonial renewal and differentiation that may also reduce the number of viable sperm and cause infertility. Vascular Endothelial Growth Factor A
(VEGFA) has been demonstrated to be important in testis differentiation—especially in cord formation and vascular development (Bott et al., 2008). Inhibition of VEGFA results in aberrant endothelial cell migration which does not allow for germ cell aggregation or formation of sex-specific vasculature (Cool et al., 2011). VEGFA is expressed in testicular endothelial cells, in proliferating germ cells, in Leydig cells and in Sertoli cells in the bovine and in the murine (Caires et al., 2009; Bott et al., 2010). Furthermore, VEGFA angiogenic and antiangiogenic isoforms have also been implicated in affecting the spermatogonial stem cell population and their ability to colonize and proliferate after germ cell transplantation into recipients (Caires et al., 2012).

Thus, to further define the mechanisms of VEGFA’s effect on spermatogenesis, the objective of my research was to examine the effects on testis morphogenesis and gene expression in male rats when given multiple injections of VEGFA164 (proangiogenic), VEGFA165b (antiangiogenic) or antiVEGFAxxxxb (antibody to all antiangiogenic isoforms) during the early postnatal stages of testis development when gonocytes resume mitosis and prior to when the spermatogonial stem niche forms (Orth et al., 2000). Because rats received a series of injections and were collected at an early age, the resulting data was indicative of transient VEGFA effects. A second project utilized Sertoli cell-specific inactivation of proangiogenic VEGFA isoforms through knockdown of its co-receptor, neuropilin-1 (NRP-1). NRP-1 binds to only proangiogenic isoforms and not antiangiogenic, and it also amplifies the signal transduction of proangiogenic isoforms actions through KDR (Soker et al., 1998). Since the mice are bred to have NRP-1 chronically eliminated, data would be indicative of long-term effects. Both studies incorporate in vivo methods of skewing the balance of proangiogenic
isoforms to antiangiogenic isoforms of VEGFA in an attempt to observe how male reproduction is affected.

**Embryonic Testis Morphogenesis**

Every embryo may be fundamentally considered a hermaphrodite since the gonads are bipotential through midgestation in mice and until approximately six weeks of gestation in humans with both reproductive tract structures present. Primordial germ cells (PGCs) originate in the yolk sac and migrate to the genital ridge where germ and somatic cells may further migrate and proliferate. Certain gene expression and cell signaling, much related to chromosomal sex, will then commit the bipotential gonad to differentiate into either a testis or into an ovary (Matzuk and Lamb, 2008; See Figure 1).

**Differentiation of Testes and Male Reproductive Tract**

The Y chromosome and corresponding male genes are responsible for initiating testis formation. The sex-determining region of the Y chromosome (SRY) is responsible for sex determination in males (Jakob and Lovell-Badge, 2011; Koopman et al., 1991); its expression upregulates SRY-related High Mobility Group (HMG) Box 9 (SOX9) and signals differentiation of the primary male somatic cells, the Sertoli (Harley et al., 2003). Upregulation of SOX-9 during male sex differentiation is conserved from avian to amphibians to mammals; however, only mammals regulate SOX-9 through expression of SRY in males.

Sertoli cells are the first testis cells to differentiate and arise from pre-somatic cells in the coelomic epithelium. They surround and amass the germ cells to form the characteristic seminiferous cords and then tubule structures (Buehr et al., 1993). Seminiferous cord formation is the first sign of testis-specific vasculature (Jost et al., 1981). Between 11.5 and 12.5 days post coitus (dpc) in mice, mesonephric vasculature
breaks down, and the endothelial cells migrate and comprise the coelomic vessel 
(Brennan et al., 2002). Sertoli cells of the testis produce factors that foster further 
development of the male reproductive tract and also maintain germ cell viability through 
the intimate contact between the somatic cells and developing sperm (Shupe et al., 2011). 
Thus, Sertoli cells are a critical cell type in the testis and the cell type that initiates testis 
differentiation. They also continue to nurture and provide the necessary nutrients for 
sperm cell development and maturation.

**Adult Testis Morphogenesis**

Adult testis function is dependent upon constant feedback and activation between 
various organs, cell types and hormones. The testis represents a conglomerate of 
mitotically- and meiotically-active germ cells as well as multiple types of somatic cells 
(Hobbs et al., 2010).

**Hypothalamic-Pituitary-Gonadal Axis**

Gonadotropin-Releasing Hormone (GnRH) is the primary hypothalamic hormone 
that initiates feedback loops and stimulates testis morphogenesis. It targets the anterior 
pituitary gonadotroph cells where follicle stimulating hormone (FSH) and luteinizing 
hormone (LH) are produced and secreted. These two hormones, then, elicit effects at the 
testis, the only place where receptors to these two hormones are present in males. LH and 
FSH each have receptors in specific cell types in the testis- Leydig cells (LH) and Sertoli 
cells (FSH) (Simoni et al., 1997).

**Cellular Roles within the Testis**

LH has receptors in interstitial Leydig cells in the testis. These are the cells that 
are found in the interstitial compartment between seminiferous tubules and that produce
testosterone. Testosterone is the primary male hormone responsible for maintenance of the male reproductive tract and various secondary sex characteristics. Testosterone can be aromatized to estrogen by Sertoli cells through the enzyme P450 aromatase. Sertoli cells have receptors for FSH. They also are the first testicular cells to differentiate and comprise the seminiferous tubules in adult males. Testosterone may also be converted to dihydrotestosterone (DHT), a hormone responsible for formation of male external genitalia (Hadley and Levine, 2007). The Leydig and Sertoli cells are the sites of testicular steroidogenesis- the process by which cholesterol can be eventually converted to testosterone and other hormones. Steroidogenesis which is dependent upon the onset of steroidogenic enzyme activity initiate at E16-17 in the mouse (El-Gehani et al., 1998).

Steroidogenesis

Cholesterol is the common precursor to all steroids and must be transported from the outer mitochondrial membrane to the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR) (Arakane et al., 1998; Kraemer et al., 2004). The enzyme P450SCC cleaves the side chain of cholesterol resulting in its conversion to pregnenolone according to Scott and others (2009). Two pathways exist that culminate in the conversion of pregnenolone to testosterone- either Δ4 or Δ5. The Δ5 pathway begins with pregnenolone being converted to 17α-hydroxy-pregnenolone by the hydroxylase activity of P450 17α-hydroxylase/17, 20 lyase (Figure 2); this is converted to dihydroepiandrosterone (DHEA) by the lyase activity of 17α-hydroxylase/17, 20 lyase. DHEA is converted to 5-androstene-3β, 17β-diol via 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3). In the Δ4 pathway, pregnenolone can be converted to progesterone via 3βHSD activity. Progesterone is converted to its intermediate, 17α-hydroxy-progesterone
and then to androstenedione; both reactions are catalyzed by the enzyme 17α-hydroxylase/17, 20 lyase. In both the Δ4 and Δ5 pathways, 17β-HSD3 cleaves androstenedione to produce testosterone (Scott et al., 2009). Testosterone is converted to DHT by P450 5α-reductase; it may also be converted to estrogen by P450 aromatase (Hadley and Levine, 2007). Adult humans predominantly utilize the Δ5 pathway while rats favor the Δ4 cascade (Hamar and Petersson, 1986; Preslock and Steinberger, 1977; Rajfer et al., 1987; Rey et al., 1995; Bell et al., 1968; Kwan et al., 1988; Samuels et al., 1975).

The somatic cells of the testis carry out steroidogenesis. The interstitial Leydig cells synthesize testosterone from progesterone produced either by the Leydig cells, themselves, or by adrenal glands and are the sites of most steroidogenesis preceding testosterone production (Scott et al., 2009). The Sertoli cells comprise seminiferous tubules (and later, cords) and aromatize testosterone to estrogen, which is critical in spermatogenesis.

**Spermatogenesis**

Spermatogenesis is the process by which postnatal gonocytes may undergo mitosis to become spermatogonial stem cells, undifferentiated spermatogonia and then may differentiate until becoming mature spermatozoa (Goertz et al., 2011). Progenitor germ cells originate from the epiblast of blastocysts and migrate to the genital ridge by 11.5 dpc in rodents (De Miguel et al., 2009). Following enclosure by Sertoli cells, rodent germ cells begin to proliferate around 13.5 dpc and are arrested as gonocytes in the G0/G1 phase a few days later (De Felici, 2009). Proliferation resumes shortly after birth (P1 in mice, P3 in rats), and gonocytes migrate from the lumen to the basement
membranes of seminiferous tubules. Here at the basement membrane, gonocytes may differentiate into SSC (Kim et al., 2010). Spermatogenesis occurs within the seminiferous epithelium in an organized and synchronized fashion. SSCs give rise to undifferentiated spermatogonia that either undergo mitotic differentiation or will proliferate; the \( A_{pr} \) (\( A_{paired} \)) subtype of spermatogonia require the ability to proliferate and renew to maintain a population of cells able to differentiate (Caires et al., 2010; Huckins, 1971a, b, c). Spermatocytes arising from differentiated spermatogonia meioitically divide; and ultimately haploid spermatids, then, mature into spermatozoa (Caires et al., 2010; Yoshida et al., 2004; Suzuki et al., 2009). This process can be observed in a stage-specific manner (spermatogenic wave) in tubule cross-sections (Russell et al., 1990).

**Formation of Spermatogonial Stem Cells/ Undifferentiated Spermatogonia**

There are no known markers that exist specifically and exclusively for spermatogonial stem cells (SSC); however, there are many genes shown to be expressed in undifferentiated spermatogonia as well as in Sertoli cells that impact SSC development. Undifferentiated spermatogonia can be classified as \( A_{stem} \) (\( A_{single} \) or \( A_s \)), \( A_{paired} \) (\( A_{pr} \)) or \( A_{aligned} \) (\( A_{al} \)) cells (Yoshida et al., 2004). The \( A_s \) spermatogonia are thought to represent the true stem cells but have demonstrated signs of heterogeneity (Yoshida et al., 2004; Suzuki et al., 2009). While different markers can be localized to be expressed in the three cell types, none have been isolated to solely \( A_s \). \( A_{pr} \) spermatogonia are characterized by two clone cells joined together by intercellular bridges (Yoshida et al., 2004), and chains of four to sixteen cells (and sometimes thirty-two) comprise the \( A_{al} \) spermatogonia (Suzuki et al., 2009). The undifferentiated cells are distinct from their differentiated counterparts; they are less advanced and undergo longer cell cycles (de
In the literature it is still unclear if Apr and Aal spermatogonia possess the ability to de-differentiate and regress to being stem cells once more (Nakagawa et al., 2007). The three cell types can be distinguished by their arrangement at the basement membrane of seminiferous tubules (Oakberg, 1971; de Rooij, 1973). Sertoli cell junctional proteins form the blood-testis barrier (prevention of autoimmune destruction of haploid germ cells) and also divide the seminiferous epithelium into basal and adluminal compartments. It is in the basal compartment that the SSC niche resides. The SSC niche is the microenvironment in which SSC and undifferentiated spermatogonia thrive and develop (Ogawa et al., 2005). Swi-independent 3a (SIN3A) is a factor produced by Sertoli cells necessary for establishing the SSC niche. Knockout of the Sin3a gene results in total male germ cell depletion by six weeks of age in mice (Payne et al., 2010). Kit Ligand (KITL), another factor produced by Sertoli cells in the testis, is important for early gonocyte migration to form nests (Manova et al., 1993). Knockout of Kitl reduces spermatogonial proliferation (Deshpande et al., 2010).

Self-renewal and Differentiation of Undifferentiated Spermatogonia

Spermatogenesis is one of the most biologically constructive processes: nearly constant spermatozoa production and, as such, fertility, in males is maintained throughout life by two events- self-renewal of spermatogonial stem cells/undifferentiated spermatogonia and cell differentiation. Self-renewal allows the stem cell pool to be replenished. The undifferentiated spermatogonia utilize that self-renewal phenomenon to replenish a pool of cells that can either renew or differentiate and ultimately be able to restore spermatogenesis entirely even when more differentiated cells are eliminated through testis damage or by in vitro methods (de Rooij et al., 1999). Differentiation of
undifferentiated spermatogonia into A1-4, intermediate (In) and then into B differentiating spermatogonia is a fate that allows for further development into mature spermatozoa (Suzuki et al., 2009). Homeostasis is ensured by the balance between self-renewal and differentiation (Hobbs et al., 2010). While there are not necessarily morphological differences between different portions of the basement membranes of seminiferous tubules, A3, Apr and Aal cluster most abundantly at the basement membranes of seminiferous tubules that border interstitium (de Rooij, 2009; Chiarini-Garcia et al., 2001, 2003; Chiarini-Garcia and Russell, 2001). Various somatic cell types contribute factors to maintenance of the SSC niche (Figure 3A; Caires et al., 2010).

**Factors Involved in Undifferentiated Spermatogonia Self-Renewal**

There are various factors known to be important for self-renewal of undifferentiated spermatogonia. See Figure 3B for a diagram of self-renewal and differentiation as well as possible factors involved in those processes. Whether genes expressed in undifferentiated spermatogonia are in true stem cells has yet to be elucidated. Glial-cell derived neurotrophic factor (GDNF) is a factor produced by Sertoli cells that mediates self-renewal (Meng et al., 2000) through binding its receptor/co-receptor complex of Ret proto oncogene/Ret tyrosine kinase (RET) and Gdnf-family receptor alpha 1 (GFRA1); both proteins comprising the receptor heterodimer are co-expressed in all three subtypes of undifferentiated spermatogonia (Suzuki et al., 2009). GDNF is stimulated by FSH (Tadokoro et al., 2002). Fibroblast growth factor 2 (FGF2) produced by Sertoli cells, may regulate renewal through GDNF, and its receptor (FGFR2) is expressed in SSC (de Rooij, 2009; Goriely et al., 2005; Simon et al., 2007).
Colony stimulating factor 1 receptor (CSF1R) has been localized in SSCs, and its ligand, CSF1, is produced by Leydig cells and has been shown to enhance proliferation of GFRA1 positive spermatogonia (Kokkinaki et al., 2009). NANOS2 is a binding protein expressed in A<sub>s</sub> and A<sub>pr</sub> spermatogonia (Suzuki et al., 2009) and is required for germline maintenance of their stem cell state or stemness (Sada et al., 2009). NANOS3 is a binding protein whose expression is overlapping but independent of that of NANOS2; it is expressed in most undifferentiated spermatogonia and even some differentiating germ cells (Suzuki et al., 2009). According to Suzuki and others (2009), NANOS3 holds value in the survival and maintenance of undifferentiated germ cells in mice. Another important factor is B-cell CLL/lymphoma 6, member B (BCL6B), and it, too, is expressed by undifferentiated spermatogonia. BCL6B is from the same family as promyleocytic zinc finger (PLZF or ZBTB16) and is one of the downstream targets of GDNF that enhances overall GDNF self-renewal activity (Oatley et al., 2006, 2007). PLZF is important for renewal of undifferentiated spermatogonia and is expressed in undifferentiated type A spermatogonia (de Rooij et al., 2009). This transcription factor, when knocked out, results in progressive germ cell loss without adversely impacting differentiation (Buass et al., 2004). PLZF modulates it effects by upregulating REDD 1 (regulated in development and DNA damage responses 1), a factor that is activated in response to various stressors to inhibit mammalian target of rapamycin complex 1 (mTORC1), a factor often important for cell growth and survival but that can exhaust the pool of available stem cells (Hobbs et al., 2011; Chen et al., 2008; Brugarolas et al., 2004; Shoshani et al., 2002). SSC renewal is stimulated by PLZF through its repression of c-Kit transcription in early spermatogonia (Filipponi et al., 2007). Ets variant gene 5 (ETV5) and lim homeobox
protein 1 (LHX1) are similar to BCL6B by being target factors of GDNF and are important for SSC maintenance (Simon et al., 2007; Oatley et al., 2006, 2007). Schlesser and others (2008) determined that ETV5 is expressed in Sertoli cells in addition to germ cells. Mice null for the TATA box-binding protein-associated factor 4b (TAF4B), which is a transcription factor, causes depletion of the SSC pool. TAF4B is expressed in spermatogonia and in spermatids (Falender et al., 2005). FOXO1 is another factor recently found to be a potential marker for development of gonocytes into SSC; immunohistochemical analysis demonstrated its translocation from the cytoplasm to the nucleus coinciding with the phenomenon. Deficiencies were seen in self-renewal in addition to diminished sperm numbers not attributed solely to apoptosis when Foxo1 was knocked out (Goertz et al., 2011).

Factors Involved in Differentiation of Spermatogonia

Yoshida and others (2004) note that undifferentiated spermatogonia are represented as c-KIT negative, meaning that c-KIT plays an important role in spermatogenic differentiation. The receptor, c-KIT is expressed in differentiating spermatogonia (AAl) through early primary spermatocytes (Schrans-Stassen et al., 1999; de Rooij et al., 2009). Stem cell factor (SCF or secreted KITL), produced by Sertoli cells, is a ligand for c-KIT and induces differentiation (de Rooij et al., 1999; Ohta et al., 2000; Laird et al., 2011). Another factor, Neurogenin3 (NEUROG3 or NGN3), is expressed in c-KIT negative undifferentiated spermatogonia. A study involving Cre-loxP approach revealed that all cells expressing NEUROG3 appeared to be the cells that gave rise to spermatogenesis by co-expressing NEUROG3 with cre-recombinase (Yoshida et al., 2004). Pellegrini and others (2003) also found that c-KIT expression was induced in c-
KIT negative cells by bone morphogenic protein 4 (BMP4). Activin A and BMP4 are suggested to be involved in differentiation because SSC maintenance was reduced when they were added to culture media (Nagano et al., 2003). The cytoplasmic-to-nuclear translocation of FOXO1 previously mentioned occurs immediately before the induction of c-KIT. Knockout of Foxo1 reduced c-KIT expression suggesting it initiates differentiation through upregulating c-KIT (Goertz et al., 2011). Knockout of Sox3 in mice demonstrated highly reduced spermatogenesis at around postnatal day 20; while spermatogenesis later seemed to be recapitulated, these findings suggest a role of SOX3 in spermatogonial differentiation (Laronda and Jameson, 2011).

**Vascular Endothelial Growth Factor A (VEGFA)**

The VEGF family is comprised of VEGFA, VEGFB, VEGFC, VEGFD and Placenta Growth Factor (PGF); VEGFA is the most prominently studied (Tammela et al., 2005). VEGFA is a 45 kDa glycoprotein coded by an 8-exon gene on chromosome 6 in humans (Plouet et al., 1989; Houck et al., 1991). VEGFA is a mitogenic (inductor of mitosis) protein that is stimulated by hypoxia, is required for angiogenesis, proliferation and migration and has non-vascular targets as well (Ferrara et al., 1989). Studies have demonstrated both *in vitro* and *in vivo* effects of hormones on VEGFA transcription. One such study utilized endometrial adenocarcinoma cells that were co-cultured with either progesterone receptor A or B (PRA or PRB) and a synthetic progestin. These cells were chosen, in part, for their known expression of VEGFA proteins so changes in expression could be measured *in vitro* (Charnock-Jones et al., 1993). *VEGFA* transcription was assayed by luciferase activity which increased when adding the receptor/progestin combination. Additionally, it was determined that there are three progesterone response
elements (PRE), and all of them are important within the VEGFA promoter to upregulate activity (Mueller et al., 2003). A similar study was conducted that incorporated the same endometrial human adenocarcinoma cells (Ishikawa cells) and a luciferase reporter in conjunction with the VEGFA promoter. The VEGFA-luciferase construct was also transfected into human primary endometrial cells. Cells were co-transfected with estrogen receptor (ER) α or β. Treatment with 17β-estradiol resulted in heightened luciferase (VEGFA) activity in the presence of either the α or β subunits and specifically through a single estrogen response element (ERE) (Mueller et al., 2000). Finally, a study sought to determine the effects of androgen replacement on vasculature and, notably, VEGFA protein and mRNA. Castrated rats (12 wks old) showed reduced expression of VEGFA. When the castrated rats were treated with testosterone propionate, and both protein and mRNA for VEGFA were increased back to basal levels in the penile corpora cavernosa (Hwang et al., 2011). Testosterone has been shown to increase VEGFA mRNA and protein, and DHT has also upregulated VEGFA in a human prostatic epithelial cell line (Haggstrom et al., 1999; Sordello et al., 1998).

VEGFA can be alternatively spliced into different isoforms (Figure 4)- both proangiogenic (promote vascular growth) and antiangiogenic (inhibit vascular growth). Proangiogenic isoforms contain an 8a exon and result from splicing at a proximal splicing site (Harper and Bates, 2008). While human VEGFA165 was the first discovered isoform and is the most potent (VEGFA164 in cattle and in rodents), splice variants at exon 6 and 7 yield additional isoforms including VEGFA121, 145, 148, 183, 189 and 203 denoted by the number of amino acids contained in the protein (Keyt et al., 1996).
Antiangiogenic isoforms of VEGFA are characterized by inclusion of an exon 8b rather than the 8a exon used by their sister prongiogenic isoforms. The inclusion of exon 8b is the result of splicing at a distal splice site. The potential function of antiangiogenic isoforms is to balance the proangiogenic isoforms and inhibit vascular growth (Haper and Bates, 2008). The sister antiangiogenic isoform to VEGFA165 is VEGFA165b in both humans and in rodents (VEGFA164b in cattle). Like the proangiogenic isoforms of VEGFA, antiangiogenic isoforms differ in their six C terminal amino acids due to differential splicing. Other antiangiogenic isoforms of VEGFA are 121b, 145b, 183b and 189b (Harper and Bates, 2008). There are roughly equal amounts of the two isoforms in many tissues (Harper and Bates, 2008). Neutralization of antiangiogenic isoforms with an antiVEGFAxxxb resulted in increased testis vascular density which suggests a major role of inhibitory VEGFA isoforms in regulating testis development (Baltes-Breitwisch et al., 2010).

Receptors for VEGFA

VEGFA binds two primary tyrosine kinase receptors as well as to a third co-receptor (Yamada et al., 2001). VEGF Receptor 2 (VEGFR2) or kinase insert domain receptor (KDR) is the primary receptor for VEGFA; binding induces greater tyrosine phosphorylation and so greater signaling (Waltenberger et al., 1994). VEGFA receptor 1 (VEGFR1) or fms-like tyrosine kinase 1 (FLT1) fosters cell survival (LeCouter et al., 2003). It has been suggested that FLT1 acts as a decoy receptor to sequester VEGFA from KDR, and it has a higher affinity for VEGFA than KDR but results in weaker signal transduction (Ferrara et al., 2004). Mice null for Flt1 die of vascular overgrowth (Kearney et al., 2002). NRP-1 is a co-receptor that augments only proangiogenic VEGFA.
signal transduction through KDR. However, NRP-1 can also modulate VEGFA binding to FLT1. NRP-1, like VEGFA and KDR, dimerizes and creates a triple dimer complex with KDR and the VEGFA protein ligand and delivers VEGFA165 to endothelial cells (Yamada et al., 2001). NRP-1, KDR and FLT1 are expressed in germ and somatic cells in bovine, human, rat and mouse testes (Caires et al., 2009; Bott et al., 2006). QRT-PCR demonstrated Kdr to be present in rat testes from E14 to E18 while Flt1 was not detected until E18. This confirms expression of both in embryonic rat testes and also that KDR is initially the receptor which VEGFA signals through (Bott et al., 2006). KDR is expressed in all endothelial cells including those found in the testis (Yamada et al., 2001).

KDR is the primary receptor to VEGFA through which most signal transduction occurs. Receptor dimers bind dimerized VEGFA protein. Antiangiogenic isoforms have little signal transduction as compared to proangiogenic isoforms of VEGFA (Woolard et al., 2004). Treatment of cultured embryonic rat testes with a KDR inhibitor resulted in inhibited cord formation compared to controls (Bott et al., 2006). Recent data in the mouse show KDR activation to correlate with undifferentiated spermatogonia proliferation as it occurs in Sertoli cells from P6 to P8 (Caires et al., 2012). KDR-LacZ mouse testes were treated with VEGFR-TKI in organ culture; β-galactosidase activity resulted in a blue color where KDR was expressed. KDR expression was found to correlate with an increase in vascular formation, the coelomic vessel and cells surrounding cords. Treatment with the VEGFA signal transduction inhibitor reduced the number of KDR-LacZ stained cells in developing testes. This suggests a role for KDR in the migration of endothelial cells from the mesonephros into the testis and possibly in endothelial proliferation and survival (Bott et al., 2010).
While the exact roles of FLT1 are largely unknown, it has been demonstrated that mice die of vascular overgrowth when Flt1 is knocked out (Kearney et al., 2002). This suggests that it acts as a decoy receptor that limits signal transduction of endogenous VEGFA through KDR. Since NRP-1 can also bind FLT1, it may be that FLT-1 is a negative regulator of angiogenesis through its competition for NRP-1 (Fuh et al., 2000). A study looking at phosphorylation of FLT1 at different time points in mice suggests it plays roles in gonocyte migration and then survival of type B differentiated spermatogonia (Caires et al., 2012).

NRP-1 is a protein of approximately 120 kDa. It contains three different domains: ala2, blb2, and c. These three domains are important for binding semaphorins (neuronal/axonal guidance), for binding VEGF (A, B, C, D and Placental Growth Factor or PGF), and for dimerization, respectively (Appleton et al., 2007). The NRP-1 b domain also houses a heparin-binding site in addition to that for VEGF. VEGFA C termini have heparin-binding sites as well; since the NRP-1 b domain’s affinity for VEGFA165 increases with heparin, it is likely that this site helps recruit ligand and co-receptor to one another (Appleton et al., 2007). Exon 8a of VEGFA binds directly to NRP-1 (Harper and Bates, 2008). VEGFA121, however, does not have a heparin binding domain. While it can bind NRP-1, it may not have its signaling enhanced by NRP-1 presentation to KDR (Soker et al., 1998; Pan et al., 2007). NRP-1 expression is broader than that of the two receptors of VEGFA; it is found in neurons as well as in endothelial cells (Grunstein et al., 2000). NRP-1 function has been implicated in both the cardiovascular and nervous systems. Spinal and cranial nerves require NRP-1 and semaphorin signaling to fasciculate (form a cluster of nerves); cardiac arteriosus and minimal vascular branching in the brain
occur when \textit{Nrp-1} is knocked out in mice (Gu \textit{et al.}, 2003). Gu \textit{et al.} (2003) also showed that NRP-1 knock-in mice lacked semaphorin binding ability. These mice survived until birth unlike mice null for \textit{Nrp-1}; this suggests a role for the co-receptor in embryonic development (Kistukawa \textit{et al.}, 1997). Caires and others (2012) demonstrated NRP-1 activity exists in gonocytes, Sertoli cells, and undifferentiated spermatogonia perinatally and gradually declines through adulthood in mice.

\textit{Signal Transduction of VEGFA}

VEGFA164 binds KDR and signals through multiple, phosphorylation-mediated pathways to elicit varied effects. Tyrosine autophosphorylation (tyrosine 1175) occurs when binding causes the kinase domain to rotate towards the inside of the dimer (Harper and Bates, 2008). The VEGFA-activated cascade for proliferation begins (after binding KDR) with phospholipase C gamma (PLC\textgamma) activating one of two second messengers (proteins that are signaled by receptor binding to activate specific cell targets)- inositol triphosphate (IP3) or diacylglycerol (DAG). IP3 diffuses into the cytoplasm to initiate the release of intracellular calcium to ensure proliferation (Faehling \textit{et al.}, 2002). DAG, however, activates protein kinase C (PKC) to activate Raf and ultimately phosphorylate mitogen-activated protein kinase (MAPK or MEK) 1/2 which phosphorylates extracellular-signal-regulated-kinase (ERK) 1/2 leading to proliferation. VEGFA164 can also signal through the phosphoinositide 3-kinase (PI3K) pathway that activates Akt (also known as protein kinase B or PKB) which results in increased gene expression and also cell survival. Although VEGFA165b binds KDR, it lacks a NRPI binding site. Binding of VEGFA165b to KDR yields much weaker signal transduction compared to VEGFA164, and may prevent binding by VEGFA164 (Figure 5). The ability of
VEGFA165b to inhibit VEGFA164 has been demonstrated (Woolard et al., 2004). A lack of a NRP-1 binding site on antiangiogenic isoforms contributes to their minimal signal transduction through KDR binding (Figure 6). The lesser signal transduction of VEGFA antiangiogenic isoforms may also be due to insufficient binding of tyrosine residues; Harper and Bates (2008) also suggest this demonstrates that antiangiogenic isoforms may not just be competitive, inactive inhibitors.

**VEGFA Function**

The VEGFA protein is a key player in tumorigenesis and in other angiogenic situations (Harper and Bates, 2008). Originally, VEGFA was discovered as a regulator of permeability and proliferation in vasculature (Grunstein et al., 2000). Grunstein and others (2000) also state that angiogenesis occurs because VEGF signaling results in chemotaxis and permeability of already-existing blood vessels as well as endothelial cell mitosis that ultimately initiates neovascularization. Confocal immunohistochemistry for vasculature markers in embryonic rat testis cultures demonstrated an extreme decrease in vascular density in tissues treated with a VEGFA inhibitor compared to unaffected controls (Bott et al., 2006).

Studies from our lab demonstrated that messenger RNA for Vegfa188, 164, and 120 were present in rat testes at embryonic day 18. Since all three isoforms were still present at P3 overlapping the time seminiferous cords form, it was suggested that VEGFA plays a role in that process (Bott et al., 2006). Treatment of embryonic rat testes in culture with a VEGFA signal transduction inhibitor (VEGFR-TKI) that would disrupt signal transduction through both KDR and FLT-1 resulted in diminished cord area and gonadal area, thus further confirming the role of VEGFA-receptor binding (to both KDR
and FLT1) in seminiferous cord formation (Bott et al., 2006). Thus, VEGFA also possesses capabilities for nonvascular roles.

Expression of VEGFA in Male Reproduction

According to Caires and others (2009), VEGFA is expressed in bovine testicular germ cells and somatic cells. Various isoforms of proangiogenic Vegfa were detected in late embryonic rat testes and were still present in early postnatal testes (Bott et al., 2006). VEGFA immunohistochemistry was performed from E14 through P5 in rat testes. Expression was first noted in Sertoli cells, interstitial cells, and some germ cell cytoplasm. VEGFA protein localization at E16 was within the cords, especially in Sertoli cells that had aggregated germ cells. VEGFA was also found in interstitium and germ cell cytoplasm. By E19, staining decreased in Sertoli cells and increased in germ cell cytoplasm. At P0, positive staining for VEGFA was similar to E19. Finally, VEGFA expression had increased in Sertoli cells and remained in specific germ cells. For these studies, a pan antibody to VEGFA was used that does not discriminate between pro- and antiangiogenic isoforms (Bott et al., 2006). Antiangiogenic Vegfa165b messenger RNA was not detected prior to cord formation in embryonic rat testes. Expression of antiangiogenic isoform Messenger RNA was found in E14 and E16 rat testes in some germ cells; at P0, they were localized to the interstitium but absent from Sertoli and germ cells (Baltes-Breitwisch et al., 2010).

VEGFA and NRP-1 Mediation of SSC Production and Model

VEGFA is known to have an impact on male germ cell development. According to Guo and others (2004), VEGFA is upregulated when A spermatogonia differentiate into type B spermatogonia. While it is unclear whether undifferentiated type A
spermatogonia are actual stem cells, we hypothesize that proangiogenic VEGFA164 is necessary for self-renewal of the SSCs and undifferentiated spermatogonia. Because we speculate that VEGFA164 is vital to the self-renewal phenomenon, we surmise that antiangiogenic VEGFA165b is necessary to stimulate differentiation or apoptosis of undifferentiated spermatogonia (Figure 7).

To determine if VEGFA isoforms affect the stem cell population, perinatal mice were injected from P3 to P5 with PBS control, 0.5 μg VEGFA164 or 0.5 μg VEGFA165b or with 1 μg IgG control, antiVEGFA164 or antiVEGFA165b. Mice were injected around the formation of SSC. Treatment with VEGFA165b resulted in increased numbers of germ cells as determined by germ cell staining, increased spermatogonia at the basement membrane and increased cord diameter in P8 mice compared to age-matched PBS controls. At the same time, germ cell loss was seen when the antibody to VEGFA165b was used. *Bcl6b* (renewal) and *Neurog3* (differentiation) expression was reduced when mice were collected at P22 in both VEGFA164 and VEGFA165b treatments compared to controls. AntiVEGFA165b treatment resulted in increased *Nanos2* (renewal) at P22 compared to controls and increased *Neurog3* compared to antiVEGFA164 treatment. Additionally, VEGFA165b treatment was shown to increase colony length and decrease colonization efficiency following germ cell transplantation (Caires et al., 2012). The treatments were similar to ours in rats with the exception that we injected an antibody to neutralize all antiangiogenic isoforms. Plus, our perinatal rats were injected when mitosis would resume and prior to the formation of SSC.

In a previous study from our laboratory a porcine Dmrt1-cre recombinase mouse line (Boyer et al., 2002) was mated to a line containing a floxed exon 3 of Vegfa (Gerber
et al., 1999). Based on the pDmrt1 expression profile, all isoforms of VEGFA (proangiogenic and antiangiogenic) would be knocked out in Sertoli cells and in a subpopulation of germ cells. Total body weights, testis weights, and epididymal weights were significantly reduced in knockout males at 6 months old, and prostate weight tended to be reduced in knockouts as well. Bcl2, a pro-survival gene, tended to be increased in pDmrt1-cre;Vegfa−/− males; and the ratio of Bcl2 mRNA relative to a pro-apoptosis counterpart, Bax, tended to also be increased. Sin3a mRNA tended to be elevated in the knockouts. Neurog3, a marker of early differentiation of undifferentiated spermatogonia, was significantly increased. While Plzf mRNA was not different between knockouts and controls, PLZF protein expression was decreased in the pDmrt1-cre;Vegfa−/− males; suggesting that there were reduced numbers of undifferentiated spermatogonia (Lu et al., submitted). These data further suggest an impact of VEGFA isoforms on the SSC niche as well as on spermatogenesis. Thus, our goal is to assay effects on the SSC niche and spermatogenesis through a NRP-1 knockout that would alter the balance of VEGFA to favor antiangiogenic isoforms. Taking together the results of the Caires and Lu studies, we propose that VEGFA164 promotes self-renewal and that VEGFA165b stimulates either differentiation or apoptosis. A model of our hypothesis and the genes that are affected are in Figure 7.

GDNF is one of the major players in self-renewal of undifferentiated spermatogonia (Meng et al., 2000). It is expressed by Sertoli cells and binds its receptor and co-receptor, RET and GFRA1, respectively to carry out its renewal abilities, and they comprise a heterodimer complex in As, A pr, and A al undifferentiated spermatogonia (Suzuki et al., 2009). BCL6B has been shown to be upregulated by GDNF, as well. So
while BCL6B is not bound by GDNF, it is a highly regulated self-renewal factor through which GDNF may further promote self-renewal (Oatley et al., 2006, 2007). Another factor that has been demonstrated in self-renewal is NANOS2. Recently, data suggest that it, too, is upregulated by GDNF. Knockout of Gfra1 resulted in a decrease in NANOS2 protein; however, intentional overexpression of Nanos2 in the Gfra1−/− mice prevented premature differentiation of undifferentiated spermatogonia and partially rescued the loss of cells when Gfra1 was ablated (Sada et al., 2012). While self-renewal is important to maintain a pool of undifferentiated spermatogonia, simultaneous differentiation is important to maintain constant spermatogenesis (Hobbs et al., 2010).

While GDNF is known to be one of the leading factors in undifferentiated spermatogonia self-renewal, a study that overexpressed GDNF embryonically increased proliferation of pancreatic islet cells and elevated transcription of the insulin gene in those cells (Mwangi et al., 2010). NEUROG3, a factor that seems to delineate early stages of differentiation and subsequent spermatogenesis (Yoshida et al., 2004), has also been demonstrated as necessary for differentiation of pancreatic progenitor and ductal cells (Gasa et al., 2004). Furthermore, Mwangi and others (2010) presented a role of GDNF in upregulating NEUROG3 expression in the pancreas of embryonic mice. Taken together, the roles of GDNF and NEUROG3 in the pancreas parallel their functions in undifferentiated spermatogonia. We propose that GDNF could be doing the same thing by stimulating NEUROG3 in undifferentiated spermatogonia to support homeostasis of self-renewal and differentiation within the testis.
Conclusion

Caires and others (2012) have demonstrated potential roles for VEGFA164 and VEGFA165b in undifferentiated spermatogonia self-renewal and differentiation, respectively. These findings complement data generated in our laboratory where genes and proteins important in undifferentiated spermatogonia self-renewal and differentiation have altered expression when both proangiogenic and antiangiogenic isoforms of VEGFA are knocked out in Sertoli cells and in a subpopulation of germ cells (Lu et al., submitted). Thus, we have sought to utilize treatments with VEGFA isoforms as well as a knockout scheme designed to selectively inactivate proangiogenic VEGFA isoforms to further elucidate the effects of VEGFA on the self-renewal and differentiation phenomena.
**Figure 1.** Genetic basis of sexual differentiation from the bipotential gonad (adapted from Matzuk and Lamb, 2008).
Figure 2. Diagram adapted from Scott et al. (2009) that depicts murine steroidogenesis.
Figure 3. A) Various somatic cell types contribute factors to maintenance of the spermatogonial stem cell (SSC) niche microenvironment (Caires et al., 2010). B) Diagram demonstrates dormant SSC, actual SSC and subtypes of undifferentiated spermatogonia, and factors that may contribute to colonization of SSC or differentiation (Caires et al., 2010).
Figure 4. Depiction of human splice variants of VEGFA proangiogenic isoforms and antiangiogenic isoforms at the mRNA and protein levels (adapted from Harper and Bates, 2008).
**Figure 5.** When proangiogenic VEGFA165 ligand binds its receptor, KDR, there is greater signal transduction than when its antiangiogenic counterpart, VEGFA165b, binds KDR (adapted from Harper and Bates, 2008).
Figure 6. NRP-1 binds only to proangiogenic isoforms of VEGFA and not to inhibitory isoforms. It acts as a co-receptor to modulate more robust signal transduction when VEGFA binds to KDR.
Figure 7. Proangiogenic VEGFA164/165 is hypothesized to drive the self-renewal of undifferentiated spermatogonia. At the same time, antiangiogenic VEGFA164b/165b is theorized to cause the germ cells to either differentiate or to undergo apoptosis (model based on findings in Cupp laboratory, University of Nebraska-Lincoln, and McLean laboratory, Washington State University-Pullman).
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Chapter 2

Vascular Endothelial Growth Factor A (VEGFA) isoforms treatment *in vivo* affects testis morphogenesis, germ cell numbers and mRNA abundance of genes that regulate survival and the self-renewal of undifferentiated spermatogonia in perinatal rat testes

Abstract

In previous studies, injections of VEGFA165b in perinatal mice inhibited the ability of spermatogonial stem cells to colonize recipient testes and also decreased colony length demonstrating that antiangiogenic VEGFA isoforms have inhibitory actions on spermatogonial stem cell renewal. Thus, we hypothesized that injections into male rat pups as gonocytes resume mitosis would alter testis composition and expression of genes that regulate survival and the SSC niche. Male rat pups were treated with either 0.5 μg or 1 μg of VEGFA164 or VEGFA165b to PBS control or 1 μg of either IgG control or antiVEGFAxxxb via intra-peritoneal (IP) injection. Pups were treated the day they were born (postnatal day 0 or P0), P1 and P2 and collected at P8. Seminiferous cord area was reduced in the 0.5 μg treatment of VEGFA165b compared to PBS control and VEGFA164; conversely, interstitial area was increased (P < 0.01). Cord area increased and interstitium decreased from VEGFA164 (1 μg) compared to both PBS and VEGFA165b injections (P < 0.01). The number of VASA-positive germ cells tended to be increased when VEGFA164 (0.5 μg) was given (P < 0.08). Testosterone (ng/mL) was reduced in males treated with 0.5 μg VEGFA165b (P < 0.05). Serum testosterone also
tended to be increased in pups injected with antiVEGFAxxxb (P < 0.07). An apoptosis gene, \( Bax \), was reduced in testes when treated with antiVEGFAxxxb compared to IgG controls (P < 0.02). While no differences were observed in its pro-survival counterpart, \( Bcl2 \), the ratio of \( Bcl2:Bax \) tended to be increased in antiVEGFAxxxb-treated pups compared to IgG controls (P < 0.08). No differences were seen in either \( Gfra1 \) or \( Gdnf \), the co-receptor and ligand, respectively, to \( Ret \), but \( Ret \) was reduced when antiVEGFAxxxb was given (P < 0.1). Like RET, \( Nanos2 \) is expressed in undifferentiated spermatogonia and is important for self-renewal of undifferentiated spermatogonia was significantly reduced in testes of rat pups given antiVEGFAxxxb versus IgG controls (P < 0.02). Taken together these data indicate that VEGFA isoforms do affect testis composition and expression of genes important in regulation of the SSC niche and in cell survival.

**Introduction**

In the rat testis gonocyte proliferation is arrested around E17. Proliferation does not resume until around P3 and continues through P5 when gonocytes migrate from the center of seminiferous cords out towards the basement membrane. It is thought that after this migration, the gonocytes differentiate into spermatogonial stem cells and/or undifferentiated spermatogonia (McGuinness and Orth, 1992a; Peters, 1970; Orth et al., 2000). Thus it is proposed that the mitotically-active gonocytes may become spermatogonial stem cells (SSC), differentiate into spermatozoa or undergo apoptosis (McGuinness and Orth, 1992a; McGuinness and Orth, 1992b; Culty, 2009). However, very little is known about how any of these processes occur and what factors regulate them.
Vascular Endothelial Growth Factor A (VEGFA) is a potent mitogen that has been shown to be important for testis formation including cord development and the derivation of sex-specific vasculature (Bott et al., 2006). Vegfa is a complex gene that can be alternatively spliced into various isoforms and is comprised of eight exons and seven introns. Splicing of the VEGFA gene may generate either proangiogenic isoforms or antiangiogenic isoforms that signal primarily through two tyrosine kinase receptors. The VEGFA isoforms and both receptors are expressed in Sertoli cells and in germ cells (Baltes-Breitswisch et al., 2010; Bott et al., 2010; Caires et al., 2012) in both rats and mice. It has been demonstrated that VEGFA is important for regulating the numbers of spermatogonial stem cells (Caires et al., 2012). Experiments utilizing perinatal male mice that had been injected with either antiangiogenic or angiogenic VEGFA isoforms at the time of SSC formation followed by the transplantation of germ cells into recipients demonstrated that antiangiogenic isoforms inhibit colonization of spermatogonial stem cells. These results suggest that treatment with antiangiogenic VEGFA isoforms inhibit the number of spermatogonial stem cells within the testis and may impair their ability to self renew or proliferate (Caires et al., 2012). Furthermore these data indicate that the balance of angiogenic versus antiangiogenic isoforms may be critical to spermatogonial stem cell renewal (Figure 1).

Therefore, we hypothesized that VEGFA164 and antiVEGFaxxxb (antibody to all ‘b’ isoforms) would increase germ cell number per cord as well as upregulate genes important in undifferentiated spermatogonia self-renewal in vivo. Furthermore, we expected in vivo treatment of male rats with VEGFA165b would reduce cord area, result in fewer germ cells per cord and would have the opposite effects on gene expression as
VEGFA164. To test this hypothesis we utilized injections of PBS (control), VEGFA164, or VEGFA165b, or IgG (control) or antiVEGFAxxxb initiated just prior to the time of the start of gonocyte proliferation to determine how an imbalance of VEGFA pro- to antiangiogenic isoform balance affects SSC development and testis morphogenesis. We hypothesized that more robust phenotypes would result from injecting VEGFA isoforms and antibodies prior at the resumption of mitosis and prior to SSC formation than treating at SSC establishment.

**Materials and Methods**

**Animals**

Sprague-Dawley rats that were pregnant upon arrival (Charles River Laboratories; Wilmington, MA) were housed with their pups at the University of Nebraska-Lincoln (UNL). Animal procedures were all approved by the UNL Institutional Animal Care and Use Committee (IACUC). Animals were humanely euthanized with a combination of carbon dioxide (CO₂) gas and cervical dislocation. Testes were collected from eight-day old male pups (P8); one was taken for histological analysis and one for RNA extraction and QPCR analysis.

**Treatments**

Pups were given one of five treatments through intra-peritoneal (IP) injections shortly after birth (P0) and once a day for the following two days (P1 and P2). Animals were selected randomly for treatment. Rats were either treated with either 0.5 μg or 1.0 μg of recombinant rat VEGF164 (n = 10, 14; rrVEGF164; R&D Systems; Minneapolis, MN; #564) or recombinant human VEGF165b (n = 10, 22; rhVEGF165b; R&D Systems; Minneapolis, MN; #CUSTOM02) with equivalent phosphate buffered saline (PBS) as a
control (n = 5, 20). Another treatment series included either 1.0 µg IgG control (n = 25; normal mouse IgG; Santa Cruz Biotechnology; #sc-2025) or antiVEGFAxxxb (n = 29; Mouse monoclonal [MRVL56/1] to VEGF165b; Abcam; San Francisco, CA; #14994-100). The antiVEGFAxxxb product is an antibody designed to eliminate all antiangiogenic (‘b’) isoforms of VEGFA. Doses were derived from previous research in the Cupp laboratory with perinatal rat ovarian and testis organ cultures taken into account that these injections were IP and in vivo (Baltes-Breitswisch et al., 2010; Artac et al., 2009; McFee et al., 2009).

Fixation, Embedding, and Staining

Testes were submerged in Bouin’s solution (750 mL picric acid, 250 mL 37-40% formalin, 50 mL glacial acetic acid) for 1 h and then rinsed in 70% ethyl alcohol in water (EtOH) until the solution was no longer yellow. Tissues were, then, rinsed for 1 h in each of three 100% EtOH baths and for 1 h in each of three baths of CitriSolv (Fisher Scientific; Fairlawn, IL). Rehydrated testes were left in liquid paraffin overnight and embedded in paraffin the following morning. The embedded tissues were sectioned into 10 slides of at least 6, 5 µm sections each. Slides were deparaffinized, rehydrated and stained with hematoxylin (VWR International; Westchester, PA) and eosin (Ricca; Arlington, TX) (H&E) for morphology.

Seminiferous Cord and Interstitial Areas

Middle sections of testes were stained with hematoxylin and eosin. Two images at 40x were taken with an Olympus BX51 microscope and Olympus DP71 camera to measure the seminiferous cord and interstitial areas. Spot Imaging Software was first used and was replaced by cellSens Standard. The Scion Image and ImageJ (updated
Scion software) programs from the National Institutes of Health (NIH) were used to outline the whole image and measure the cord area of testis sections. Seminiferous cord areas were subtracted from the total area to determine the area of the interstitium. Areas were recorded in pixels, and image size in pixels was adjusted for the updated imaging software. Two technicians performed the measurements for each image, and values were averaged for each sample and across treatment. Values were recorded for subsequent statistical analysis.

**VASA Immunohistochemistry and Germ Cell Count**

Middle sections of testes were used for immunohistochemical analysis. Slides were deparaffinized, rehydrated and microwaved for 15 min to boil in 0.01 M sodium citrate for antigen retrieval. Slides were cooled for 1 h at room temperature and then rinsed in 3% hydrogen peroxide (H₂O₂) in methanol for 20 min and then in 1x PBS 3 times for 2 min each. A Pap pen (Research Products International Corp.; Mount Prospect, IL) was used to isolate a negative control section and treatment section. Sections were blocked with 10% normal goat serum (NGS; PAA Laboratories, Inc.; Ontario, Canada) diluted in PBS for 30 min. NGS was left on the negative control section to prevent drying out but removed from treatment sections. A 1:500 dilution of a VASA (anti-DDX4 rabbit polyclonal Ab; Abcam; San Francisco, CA; catalog #ab13840) primary antibody diluted in PBS was left on the treatment sections overnight while the slides were covered and stored at 4°C; VASA is marker that expresses in germ cells. Next, slides were rinsed 3x for 2 min in PBS. A secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Inc.; Burlingame, CA) diluted in PBS at 1:300 was placed on all sections while slides incubated for 30 min. Two drops of Vectastain ABC Kit (Goat IgG; Vector
Laboratories, Inc.; Burlingame CA) in PBS were added to each section after another rinsing in 3 PBS washes for 2 min each; this was 20 min incubation. Vector NovaRED Substrate Kit (Vector Laboratories, Inc.; Burlingame, CA) was added to sections following another 3x 2 min PBS rinse. The solution was left on for 1 min, and slides were placed in distilled water to halt the reaction. Slides were blotted between all stages after standing in the distilled water; they were cover-slipped after the addition of mounting media (Abcam; San Francisco, CA). Stained and covered slides were covered and stored at 4°C. Two 40x images were taken. Red, ring-shaped staining represented VASA expression. The number of cells per cord for each of two images per animal were counted by two technicians and averaged in animal and again within treatment. Germ cells per cord were recorded for statistical analysis.

**RNA Extraction and Quantification**

One testis was collected from each pup for RNA extraction, reverse transcription to cDNA and QPCR analysis. Testes were stored in Tri-Reagent (Sigma; St. Louis, MO) and homogenized using a 1 CC syringe and increasing gauge needles as the tissue surface area decreased. Subsequent RNA extraction began at room temperature with adding 0.2 mL of chloroform to each sample, vortexing for 15 sec and letting them set for 5 min. Samples were then centrifuged at 4°C for 10 min at 12,000 g. Once the supernatants were removed and transferred to new microcentrifuge tubes, 0.5 mL isopropyl alcohol was added to each sample. Samples were vortexed and allowed to sit at room temperature for 5 min; they were again centrifuged for 10 min at 12,000 g at 4°C. 1 mL 75% EtOH in 25% diethyl-pyrocarbonate (DEPC)-treated water was added. Samples were vortexed and centrifuged at 4°C for 5 min at 12,500 g. Supernatants were poured off; samples were
allowed to air dry; and 20 μL of DEPC water were added to each ethanol pellet. 1 μL was removed to quantify the concentration of RNA (ng/mL) present via a Lambda EZ 150 Nano Drop Spectrophotometer (Perkin Elmer). Absorbencies at both 260 nm and 280 nm were measured, and a ratio of the two was calculated to determine concentration of RNA. Samples were stored at -80°C until reverse transcription was performed.

Reverse Transcription to cDNA

DEPC water was added to enough concentrated RNA to reach a 15 μL reaction mixture for each sample and to result in 5 μg of cDNA. A master mix was added to each sample consisting of 5 μL DNase (Promega; Madison, WI; Catalog #M6101) and 5 μL 5x First Strand Buffer (Invitrogen) to eliminate any DNA contamination. Samples were incubated in a PTC-200 thermal cycler (MJ Research; Quebec, Canada) for 30 min at 37°C. 1 μL DNase Stop Solution (Promega) was added to each sample, and samples were incubated at 65°C for 10 min. To conclude the DNase treatment reaction, 1 μL each sample was pipetted into a new tube with 15 μL DEPC water to represent ‘No Reverse Transcription (No RT)’ controls. These samples were pooled and demonstrated whether there was residual genomic DNA when Real-Time PCR was performed; Gapdh was the sole QPCR for the No RTs. A master mix comprised of 2 μL DEPC water, 1 μL Random Primers (Promega; Catalog #C118A), and 1 μL 10 mM deoxynucleotide triphosphates (dNTPs; Promega; U151B) was added. Samples were incubated for 5 min at 65°C and then chilled on ice. Subsequently, a second master mix was added with 4 μL 5x First Strand Buffer (Invitrogen Life Technologies; Carlsbad, CA), 1 μL 0.1 M DL-dithiothreitol (DDT; Invitrogen), 1 μL RNaseOUT (Invitrogen), and 1 μL of a reverse transcriptase, Super Script III (Invitrogen). Each sample was briefly centrifuged. Samples
were incubated at 50°C for 50 min, 70°C for 5 minutes and again chilled on ice. They were briefly centrifuged and had 1 μL RNase H (Invitrogen) added to further eliminate RNA contamination. After a 20 min incubation at 37°C, samples were stored at -20°C until used for QPCR.

**Real-Time PCR (QPCR)**

Master mixes were added to 0.5 μL cDNA template in Quantitative Real-Time PCR (QPCR). Taqman Universal PCR and Power SYBR Green Master Mixes were used (Applied Biosystems; Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was the housekeeping gene to which all others were normalized as its expression is constitutive; rodent Gapdh primers and probe came from a predesigned kit. See Table 1 for kits and sequences for genes in this study. QPCR was performed using a 384-well plate, and samples were run in triplicate in separate consecutive wells. The machine (7900, Applied Biosystems) cycled through different stages- 50.0°C for 2 min, 95°C for 10 min, 95°C for 15 sec and then 60°C for 1 min (repeated for as many cycles as the user sets), and finally 95°C for 15 sec. Different dilutions of cDNA from similar tissues as the unknowns were used as standards. An arbitrary value was given to the undiluted standard, and values were ascribed to subsequent dilutions. Those values were plotted against the cycle threshold value where amplification is first detected to synthesize the standard curve. TaqMan Master Mix was used for Gapdh, Bcl2, Bax, Gdnf, Ret and Sin3a. In addition to the template, master mixes included 0.25 μL, 20 μM each of forward and reverse primers, 1 μL 2 μM probe, 5 μL 2x TaqMan and 3 μL Millipore water. Genes that were measured via a TaqMan Gene Expression Assay (Applied Biosystems) required 5 μL TaqMan Master Mix, 4 μL Millipore water, and 0.5 μL of the all-inclusive probe
and primer kit. SYBR Green was used for Nanos2, Bcl6b, Gfrc1, Vegfa164, Vegfa165b, Plzf, Casp3, Casp9, Hsd17b3 and Cyp17. Since differences in messenger RNA abundance were seen primarily amongst the IgG and antiVEGFAxxxb treatments, Sin3a, Casp3, Casp9, Hsd17b3 and Cyp17 were run only on those samples. Primers for use with SYBR Green were designed with Primer Express 3.0 software (Applied Biosystems) and synthesized at Integrated DNA Technologies, Inc. (Coralville, IA). Those reactions required 5 μL of Power SYBR Green, 0.3 μL, 20μM each of forward and reverse primers, and 3.9 μL Millipore water. Genes that required SYBR to measure were also plotted on a dissociation curve to ensure that there was not primer dimerization due to the lack of a probe. The dissociation curve added a new stage for the machine to cycle through- 95°C for 15 sec, 60°C for 15 sec and then finished again at 95°C for 15 sec. Unknown values were plotted along the standard curve. Average quantities for unknowns were divided by average quantities of Gapdh for the same samples to derive relative quantities. All individual quantities were divided by the means for controls which resulted in the controls equaling ‘one’ and treatments being represented as fold changes. Data were then analyzed statistically.

**Blood Collection and Hormone Quantification**

Trunk blood was collected at the time of euthanasia and placed in microcentrifuge tubes containing 20 μL 30% potassium EDTA (Fisher Scientific Co.; Fair Lawn, NJ). Blood was centrifuged immediately after collection for 12,000 g at 4°C for 10 min. Plasma was transferred to a new tube and stored at -80°C. Testosterone concentration was determined by an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Alpha Diagnostics International, Inc.; San Antonio, TX; Catalog #1880). Quantities were determined by the
manufacturer’s protocol. The standard curve consisted of samples with concentrations of 0 ng/mL, 1 ng/mL, 2.5 ng/mL, 10 ng/mL and 20 ng/mL. The sensitivity of the ELISA was 0.125 ng/mL. Data were transformed using Prism in order to be represented graphically.

Statistical Analysis

All data were analyzed in JMP, a business unit of SAS (SAS Institute; Cary, NC). The VEGFA164- and VEGFA165b-treated samples with PBS as a control were analyzed via a t-test to compare all pairs. The Dunnett’s procedure was used to analyze the anti-VEGFAxxxb and IgG treatments as it compares treatments back to a set control. Differences were considered significant at P<0.05. Data were considered to be approaching significance if P<0.1 but P>0.05.

Results

Seminiferous Cord Area, Interstitial Area and Histology

Two different doses were used in this study to evaluate a possible dose response. The seminiferous cord areas and interstitial areas were measured in pixels and represented graphically as fold changes compared to controls (set at 1) for each series of treatments. Figure 2 shows the measurements of seminiferous cord and interstitial areas as well as testis histology for animals given PBS or 0.5 μg of either VEGFA164 or VEGFA165b. Injection of VEGFA165b significantly reduced cord area (0.95±0.011, P < 0.01; Fig. 2A) compared to PBS (1±0.0046) and VEGFA164 (0.99±0.0078).

Subsequently, the interstitial area was increased in the VEGFA165b treatment (1.23±0.05, P < 0.01; Fig. 2C) compared to and PBS (1±0.02) and VEGFA164 (1.063±0.035). Hematoxylin and eosin staining of testis cross-sections demonstrates the
decreased seminiferous cord area and increased interstitium in VEGFA165b-treated rats (Fig. 2E) compared to PBS (Fig. 2B) and VEGFA164 (Fig. 2D).

Administering 1 μg of VEGFA164 resulted in an increased seminiferous cord area in rat testes (1.038±0.016, P < 0.01; Fig. 3A) compared to PBS (1±0.0087) and VEGFA165b (1.002±0.0079). As shown in Figure 3C, the interstitium was decreased in conjunction with increased cord area for treatment of 1 μg VEGFA164 (0.82±0.061, P < 0.01) compared to PBS (1±0.03) and VEGFA165b (1±0.033). Representative histology is included for testes of pups treated with VEGFA164 (Fig. 3D), PBS (Fig. 3B) and VEGFA165b (Fig. 3E).

Treatment with IgG or antiVEGFAxxxb did not alter testis composition (Fig. 4A and C). While there were no differences for any testis morphogenic characteristics in the antibody-treated testes compared to control IgG, images are included (Fig. 4B and D).

**VASA Immunohistochemistry and Germ Cell Counts**

Germ cells were marked by VASA staining and were counted per cord. The number of VASA-positive germ cells tended to increase from treatment with 0.5 μg VEGFA164 (1.45±0.12, P < 0.08; Fig. 5A) compared to PBS (1±0.026) but was not different from VEGFA165b. Images depicting VASA immunohistochemistry are included for PBS (Fig. 5B), VEGFA164 (Fig. 5C) and VEGFA165b (Fig. 5D).

No differences were observed in germ cell numbers for pups treated with PBS or 1 μg VEGFA164 or VEGFA165b (Fig. 6A). Regardless, images showing VASA staining are included for PBS (Fig. 6B), VEGFA164 (Fig. 6C) and VEGFA165b (Fig. 6D). Treatment with either IgG or antiVEGFAxxxb did not yield any differences in the
number of VASA-positive germ cells per cord (Fig. 7A). Images from an IgG-treated testis (Fig. 7B) and one treated with antiVEGFAxxxb (Fig. 7C) are included.

**QPCR of Cell Survival/Apoptosis Genes**

*Bcl2*, a gene important for promoting survival, was not significantly different in any of the treatments. However, while *Bax* mRNA abundance was not different for any of the VEGFA isoforms treatments, treatment with antiVEGFAxxxb reduced expression of *Bax* (0.51±0.11, *P* < 0.02; Fig. 8H) compared to IgG control (1±0.15). The ratio of *Bcl2:Bax* tended to be increased in testes of rats that received antiVEGFAxxxb (3.19±1.05, *P* < 0.08; Fig. 8I) compared to IgG (1±0.14). No other differences in *Bax* and *Bcl2* mRNA abundance were observed; likewise, no differences were seen in the expression of *Casp3* and *Casp9* (Fig. 8).

**QPCR of SSC Self-Renewal Genes**

We were interested in determining how VEGFA isoforms affect genes regulating the spermatogonial stem cell niche since previously our lab and collaborators have demonstrated differences in number of stem cells several days after *in vivo* injection with VEGFA isoforms. There were no differences in either *Gdnf* mRNA abundance or its co-receptor, *Gfra1*. However, expression of *Ret* tended to be decreased in rat testes when antiVEGFAxxxb was administered (0.7±0.13, *P* < 0.1; Fig. 9H) compared to IgG controls (1±0.12). In addition to *Gdnf, Ret* and *Gfra1*, we found no differences in the measured expression of *Sin3a* (Fig. 9J), another gene expressed by Sertoli cells important for establishment of the SSC niche (Payne *et al.*, 20120).

We examined other genes expressed by undifferentiated spermatogonia that are important for self-renewal- *Nanos2, Bcl6b* and *Plzf* (Oatley *et al.*, 2006, 2007; de Rooij *et
al., 2009; Suzuki et al., 2009). Messenger RNA abundance for Nanos2 was significantly reduced in testes of rats treated with antiVEGFAxxxb (0.47±0.11, P < 0.02; Fig. 10G) compared to treatment with IgG (1±0.24). No differences were seen from any other treatment or in Bcl6b or Plzf.

**QPCR of VEGFA Isoforms**

We looked at VEGFA isoforms, genes regulating cell apoptosis and genes important in regulating the spermatogonial stem cell niche. Messenger RNA for Vegfa164 was not increased in testes of pups treated with 1 μg VEGFA164 (2.28±0.79, P < 0.02; Fig. 11D) compared to both PBS (1±0.13) and 1 μg VEGFA165b (0.84±0.1). Conversely, Vegfa164 expression was reduced in testes of rats treated with antiVEGFAxxxb (0.56±0.097, P < 0.05; Fig. 11G) compared to IgG controls (1±0.2). As pictured in Figure 11E, Vegfa165b tended to be increased upon a 1 μg injection of VEGFA164 (1.47±0.49, P < 0.07; Fig. 11F) compared to the same of VEGFA165b (0.74±0.15), but there was no difference in either from PBS (1±0.13). While antiVEGFAxxxb treatment resulted in decreased Vegfa164, it also caused a tendency for decreased Vegfa165b expression (0.58±0.11, P < 0.08; Fig. 11H) relative to IgG controls (1±0.21). No differences were seen in the ratio of Vegfa164:Vegfa165b from any treatment and were no differences in any mRNA abundance resulting from injecting 0.5 μg VEGFA164 or VEGFA165b compared to PBS (Fig. 11).

**Serum Testosterone and Steroidogenic Genes**

Testosterone was significantly reduced in rat pups treated with 0.5 μg VEGFA165b (0.34±0.097 ng/mL) compared to PBS controls (PBS: 0.88±0.37 ng/mL), but VEGFA164 treatment was not different (P < 0.05; Fig. 12A). Serum testosterone
tended to be elevated in pups treated with antiVEGFAxxxb (0.44±0.06 ng/mL) compared to IgG controls (0.32±0.03 ng/mL, P < 0.07; Fig. 12E). When pups were given PBS or either 1 μg VEGFA164 or VEGFA165b, testosterone was not significantly different (Fig. 12C).

Because of the differences in testosterone, we investigated the expression of genes in the steroidogenic pathway. Messenger RNA abundance for Cyp17 tended to be reduced in testes of pups treated with antiVEGFAxxxb (0.67±0.12) compared to control IgG treatment (1±0.16, P < 0.1; Fig. 12B). Expression of Hsd17b3 did not differ (Fig. 12D).

Discussion

Our data demonstrate an effect of VEGFA isoforms on testis morphogenesis and on expression of genes that regulate cell survival and the SSC niche as extensions to our previous research. VEGFA165b injection (0.5 μg) decreased seminiferous cord area and increased interstitial area compared to both controls and VEGFA164 treatment. 1 μg VEGFA164 increased cord area and reduced the interstitium compared to PBS control and VEGFA165b. The number of VASA-positive germ cells per cord tended to be increased in testes of rats given 0.5 μg VEGFA164 compared to PBS controls. Caires and others (2009) showed that addition of VEGFA isoforms increased germ cell survival and proliferation in bovine testis tissue explants.

Treatment with an antibody to all antiangiogenic isoforms resulted in decreased Bax expression, and BAX is a factor that fosters apoptosis. As a result, the ratio of Bcl2, a pro-survival gene, to Bax tended to be increased also in antiVEGFAxxxb treated rats’ testes compared to IgG. While we have not yet investigated whether or not apoptosis is
occurring, the gene expression suggests that elimination of antiangiogenic isoforms of VEGFA could support cell survival (Hiklin and Ellis, 2005; Caires et al., 2009).

AntiVEGFAxxxb treatment reduced Nanos2 compared to controls and also tended to reduce Ret expression. Both genes are important for undifferentiated spermatogonia self-renewal (Oatley et al., 2006, 2007; Suzuki et al., 2009). While Gdnf was unaffected, it is a major self-renewal factor produced by Sertoli cells that binds RET and also upregulates NANOS2 in undifferentiated spermatogonia (Sada et al., 2012). Caires and others (2012) proposed that VEGFA164 is important for self-renewal of undifferentiated spermatogonia, so it was not surprising that Ret was affected by the increase in endogenous VEGFA164 and other proangiogenic isoforms in the antiVEGFAxxxb treatment. VEGFA164 has been demonstrated to upregulate GDNF and to increase phosphorylation of RET in kidneys (Tufro et al., 2007). Although we did not see a difference in Gdnf in this study, the tendency for reduced Ret is not surprising. The tendency for its reduction as well as that, treating with antiVEGFAxxxb, reduced Nanos2 suggests more of our proposed homeostatic mechanism. Neutralizing all antiangiogenic isoforms would potentially favor self-renewal of undifferentiated spermatogonia and overwhelmingly because of all the relative endogenous VEGFA proangiogenic isoform activity. Thus, genes important for self-renewal can be downregulated to remain equilibrated with differentiation. While expression of Gdnf was not different, reductions in both Ret and Nanos2 could be indicative of a lack of GDNf signaling. This is further strengthened by NANOS2 being important for maintaining stemness or the ability of a cell to remain in a stem cell state (Sada et al., 2009)
The balance of pro- to antiangiogenic VEGFA isoforms is also seen in how the antibody treatment, while it did reduce mRNA abundance for Vegfa165b, resulted in reduced Vegfa164 expression. Surprisingly, VEGFA164 (1 μg) resulted in increased mRNA for Vegfa164 compared to PBS and VEGFA165b. Also striking was that VEGFA165b (1 μg) treatment resulted in a surprising tendency for reduced Vegfa165b compared to VEGFA164 but was not different from controls. At the same time, antiVEGFAxxxb injections yielded decreased Vegfa164 and a tendency for reduced Vegfa165b compared to IgG controls. Thus, it would seem that neutralization of all antiangiogenic isoforms impairs Vegfa165b transcription and that proangiogenic Vegfa164 transcription is reduced to still preserve the delicate balance between the two sister isoform families.

Finally, the reduction in Bax in antibody-treated rat pups as well as the tendency in the increase in the Bcl2:Bax ratio suggests a role in proangiogenic isoforms driving survival whereas their hindrance in mice favor apoptosis as far as gene expression is concerned. Previous data have exhibited the role of proangiogenic isoforms of VEGFA in promoting cell survival (Hiklin and Ellis, 2005; Caires et al., 2009).

Testosterone was significantly reduced from 0.5 μg treatment of VEGFA165b compared to PBS controls in this study. At the same time, antiVEGFAxxxb tended to increase testosterone concentrations in the serum compared to IgG treatment. These data are in agreement, and since rats received IP injections, multiple cell types could have been affected. VEGFA is expressed in Leydig cells; thus, it could be that VEGFA165b antagonizes testosterone synthesis and that the relative increase of proangiogenic VEGFA stimulates testosterone production. Additionally, we quantified the mRNA abundance of
steroidogenic genes. While *Hsd17b3*, an enzyme that converts androstenedione to testosterone, did not differ, *Cyp17* tended to be reduced in testes of rats treated with antiVEGFAxxxb. This gene is important to convert progesterone and ultimately produce androstenedione (Nakajin and Hall, 1981). In a study by Kostic and others (2011) in which testosterone was administered at elevated levels over time to adult rats as well as to Leydig cells from intact rats *in vitro*, expression of many steroidogenic enzymes, including *Cyp17*, was reduced.

In the current study, a transient treatment of VEGFA isoforms had dramatic effects on testis morphogenesis and on expression of genes that regulate both the SSC niche and cell survival. Treatment with VEGFA164 or VEGFA165b seems to be the most effective means of altering seminiferous cord and interstitial areas while complete removal of antiangiogenic isoforms of VEGFA appears to have the greatest effects on gene expression. Taking into account that cord area was increased by VEGFA164 (1 μg), that VEGFA164 (0.5 μg) tended to increase VASA-positive germ cells per cord, and that antiVEGFAxxxb treatment reduced *Bax* expression and tended to increase *Bcl2:Bax*, VEGFA proangiogenic isoforms may be enhancing survival when perinatal rats are treated at the resumption of mitosis in gonocytes. Thus VEGFA isoforms are regulators of testis morphogenesis and undifferentiated spermatogonia homostasis in the rat. Altering the balance of VEGFA isoforms can affect genes that regulate the stemness of spermatogonia and affect their potential survival and function.
**Figure Legends**

**Table 1.** Quantitative PCR primers, probes and kits.

**Figure 1.** Treatment scheme for injecting perinatal rat pups and early testis differentiation and germ cell development.

**Figure 2.** Testis seminiferous cord (A) and interstitial areas (C) measured in pixels for pups treated with PBS or 0.5 μg VEGFA164 or VEGFA165b. Histology is represented for PBS-treated (B), VEGFA164-treated (D) and VEGFA165b-treated (E). Significance was set at $P < 0.05$. For PBS, $n = 10$; for VEGFA164, $n = 10$; and for VEGFA165b, $n = 10$.

**Figure 3.** Testis seminiferous cord (A) and interstitial areas (C) measured in pixels for pups treated with PBS, or 1 μg VEGFA164 or VEGFA165b. Histology is represented for PBS-treated (B), VEGFA164-treated (D) and VEGFA165b-treated (E). Significance was set at $P < 0.05$. For PBS, $n = 20$; for VEGFA164, $n = 14$; and for VEGFA165b, $n = 22$.

**Figure 4.** Testis seminiferous cord (A) and interstitial areas (C) measured in pixels for pups treated with IgG or 1 μg antiVEGFAxxxb. Histology is represented for IgG-treated (B) and antiVEGFAxxxb-treated (D). For IgG, $n = 25$; and for antiVEGFAxxxb, $n = 29$.

**Figure 5.** Immunohistochemistry for VASA based on the numbers of VASA-positive germ cells per cord for pups treated with PBS or 0.5 μg VEGFA164 or VEGFA165b (A). Images are presented with VASA staining for PBS-treated (B), VEGFA164-treated (D) and VEGFA165b-treated (E). Significance was set at $P < 0.05$. Data were considered tending toward significance when $0.1 > P > 0.05$. For PBS, $n = 5$; for VEGFA164, $n = 10$; and for VEGFA165b, $n = 10$.

**Figure 6.** Immunohistochemistry for VASA based on the numbers of VASA-positive germ cells per cord for pups treated with PBS or 1 μg VEGFA164 or VEGFA165b (A). Images are presented with VASA staining for PBS-treated (B), VEGFA164-treated (C) and VEGFA165b-treated (D). For PBS, $n = 20$; for VEGFA164, $n = 14$; and for VEGFA165b, $n = 22$.

**Figure 7.** Immunohistochemistry for VASA based on the numbers of VASA-positive germ cells per cord for pups treated with IgG or 1 μg antiVEGFAxxxb (A). Images are presented with VASA staining for IgG-treated (B) and antiVEGFAxxxb-treated (C). For IgG, $n = 25$; and for antiVEGFAxxxb, $n = 29$.

**Figure 8.** Messenger RNA abundance of genes involved in survival and apoptosis. Bcl2 mRNA is depicted for pups treated with A) PBS or 0.5 μg VEGFA164 or VEGFA165b, D) PBS, or 1 μg VEGFA164 or VEGFA165b and G) IgG or 1 μg antiVEGFAxxxb. Bax expression is presented for pups treated with B) PBS or 0.5 μg VEGFA164 or
VEGFA165b, E) PBS, or 1 μg VEGFA164 or VEGFA165b and H) IgG or 1 μg antiVEGFAxxxxb. The ratio of Bcl2:Bax is shown for rat pups treated with C) PBS or 0.5 μg VEGFA164 or VEGFA165b, F) PBS, or 1 μg VEGFA164 or VEGFA165b and I) IgG or 1 μg antiVEGFAxxxxb. Casp3 expression (J) and mRNA abundance for Casp9 (K) in testes of IgG-treated or antiVEGFAxxxxb-treated pups is shown. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P < 0.05. Data were considered tending toward significance when 0.1 > P > 0.05. For PBS, n ≥ 1; for VEGFA164, n ≥ 3; for VEGFA165b, n ≥ 3; for IgG, n ≥ 12; and for antiVEGFAxxxxb, n ≥ 11.

**Figure 9.** Messenger RNA abundance of Sertoli cell-expressed self-renewal gene Gdnf, its receptor/co-receptor components, Ret and Gfra1. Both genes comprising the receptor/co-receptor complex are expressed in undifferentiated spermatogonia. Gdnf mRNA is depicted for pups treated with A) PBS or 0.5 μg VEGFA164 or VEGFA165b, D) PBS, or 1 μg VEGFA164 or VEGFA165b and G) IgG or 1 μg antiVEGFAxxxxb. Ret expression is presented for pups treated with B) PBS or 0.5 μg VEGFA164 or VEGFA165b, E) PBS, or 1 μg VEGFA164 or VEGFA165b and H) IgG or 1 μg antiVEGFAxxxxb. Gfra1mRNA abundance is shown for rat pups treated with C) PBS or 0.5 μg VEGFA164 or VEGFA165b, F) PBS, or 1 μg VEGFA164 or VEGFA165b and I) IgG or 1 μg antiVEGFAxxxxb. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P < 0.05. Data were considered tending toward significance when 0.1 > P > 0.05. For PBS, n ≥ 3; for VEGFA164, n ≥ 5; for VEGFA165b, n ≥ 4; for IgG, n ≥ 16; and for antiVEGFAxxxxb, n ≥ 10.

**Figure 10.** Expression of other genes expressed in germ cells that are important for self-renewal but not part of the Gdnf family. Nanos2 mRNA is represented for pups treated with A) PBS or 0.5 μg VEGFA164 or VEGFA165b, D) PBS, or 1 μg VEGFA164 or VEGFA165b, and G) IgG or 1 μg antiVEGFAxxxxb. Bcl6b expression is graphed for pups that were given B) PBS or 0.5 μg VEGFA164 or VEGFA165b, E) PBS, or 1 μg VEGFA164 or VEGFA165b, and H) IgG or 1 μg antiVEGFAxxxxb. Plzf mRNA is shown for pups treated with C) PBS or 0.5 μg VEGFA164 or VEGFA165b, F) PBS, or 1 μg VEGFA164 or VEGFA165b, and I) IgG or 1 μg antiVEGFAxxxxb. Significance was set at P < 0.05. For PBS, n ≥ 2; for VEGFA164, n ≥ 4; for VEGFA165b, n ≥ 7; for IgG, n ≥ 14; and for antiVEGFAxxxxb, n ≥ 15.

**Figure 11.** Gene expression of Vegfa isoforms. Vegfa164 mRNA is depicted for pups treated with A) PBS or 0.5 μg VEGFA164 or VEGFA165b, D) PBS, or 1 μg VEGFA164 or VEGFA165b and G) IgG or 1 μg antiVEGFAxxxxb. Vegfa165b expression is presented for pups treated with B) PBS or 0.5 μg VEGFA164 or VEGFA165b, E) PBS, or 1 μg VEGFA164 or VEGFA165b and H) IgG or 1 μg antiVEGFAxxxxb. The ratio of Vegfa164:Vegfa165b is shown for rat pups treated with C) PBS or 0.5 μg VEGFA164 or
VEGFA165b, F) PBS, or 1 μg VEGFA164 or VEGFA165b and I) IgG or 1 μg antiVEGFAxxxb. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P < 0.05. Data were considered tending toward significance when 0.1 > P > 0.05. For PBS, n ≥ 1; for VEGFA164, n ≥ 2; for VEGFA165b, n ≥ 4; for IgG, n ≥ 12; and for antiVEGFAxxxb, n ≥ 14.

**Figure 12.** Serum testosterone concentrations (ng/mL) from rat pups treated with A) PBS or 0.5 μg VEGFA164 or VEGFA165b, C) PBS, or 1 μg VEGFA164 or VEGFA165b and E) IgG or 1 μg antiVEGFAxxxb and mRNA abundance of genes involved in the steroidogenic pathway of IgG- or antiVEGFAxxxb-treated animals- B) Cyp17 and D) Hsd17b3. Significance was set at P < 0.05. Data were considered tending toward significance when 0.1 > P > 0.05. For PBS, n ≥ 5; for VEGFA164, n ≥ 10; and for VEGFA165b, n ≥ 10.

**Figure 13.** Model proposing influence of VEGFA on undifferentiated spermatogonia renewal and differentiation.
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**Table 1**
Figure 1
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Chapter 3

Neuropilin-1 (NRP-1) loss in Sertoli cells alters expression of genes necessary for cell survival and for renewal and differentiation of the spermatogonial stem cell (SSC) niche and may result in subfertility

Abstract

Vascular Endothelial Growth Factor A (VEGFA) isoforms alter the renewal and differentiation of the spermatogonial stem cell niche but the exact mechanisms are unknown. Therefore, we hypothesized that inactivating VEGFA angiogenic isoforms by eliminating NRP-1, a co-receptor only binding VEGFA angiogenic isoforms, in Sertoli cells would suppress genes regulating the SSC niche and impair testis function. We generated \textit{Amhr2-Cre;Nrp-1}^+/− male mice and collected them at 79 d when testis (P < 0.003) and prostate (P < 0.03) weight were increased compared to controls. Messenger RNA abundance of \textit{Bcl2}, a pro-survival gene, was 4.4-fold less (P < 0.03) in \textit{Amhr2-Cre;Nrp-1}^+/− but there was no differences in other apoptotic genes or TUNEL assays. Sertoli cell specific genes \textit{Sin3a}, was reduced by 6.6-fold (P < 0.04,) and \textit{Gdnf}, was reduced by 14.8-fold (P < 0.05), while, \textit{Kitl}, tended to be reduced 4.3-fold (P < 0.1) in \textit{Amhr2-Cre;Nrp-1}^+/− males. The co-receptor to GDNF, \textit{Gfra1}, was not different; however, its receptor \textit{Ret} tended to be reduced in knockouts (2-fold, P < 0.09). Germ cell specific genes such as \textit{Neurog3} a germ cell transcription factor important for early differentiation, was reduced by 7.9-fold (P < 0.01) compared to controls. Surprisingly, there was a 4.4-fold \textit{increase} in the amount of \textit{Plzf} mRNA in \textit{Amhr2-Cre;Nrp-1}^+/− testes; (P < 0.002) and an increase in PLZF (P = 0.0004) positive staining in testis tubules suggesting there was increased number of undifferentiated SSC’s in the \textit{Amhr2-Cre;Nrp-1}^+/− testes. For fertility
tests, knockout males, when mated to control females, produced fewer pups in the first litter \((P = 0.0001)\) and overall litters \((P = 0.03)\), tended to require more days between parturitions \((P < 0.1)\) and resulted in fewer pups surviving to weaning \((P = 0.0003)\) than controls. Thus, in 78.6 d-old \(Amhr2-Cre;Nrp-1^{-/-}\) testes, reduced VEGFA proangiogenic actions reduced mRNA abundance for some critical SSC renewal genes while increasing mRNA abundance for \(Plzf\). We hypothesize these divergent actions of VEGFA isoforms may indicate that a balance of VEGFA angiogenic and antiangiogenic isoforms are necessary for appropriate expression of genes regulating the SSC niche to maintain fertility.

**Introduction**

VEGFA angiogenic isoforms are upregulated in the spermatogonial A-to-B transition during spermatogenesis (Guo *et al.*, 2004). While VEGFA angiogenic and antiangiogenic isoforms and receptors have been established to be present during testis formation, their exact role in early germ cell development has not been thoroughly investigated. VEGFA and KDR are both present during testis morphogenesis and are expressed by Sertoli cells and expressed in germ cells. NRP-1 is also present in Sertoli cells and in germ cells (Bott *et al.*, 2006; Caires *et al.*, 2009). Recently, our laboratory, in collaboration with others, has demonstrated that the balance of VEGFA angiogenic versus antiangiogenic isoforms may determine spermatogonial stem cell fate decisions such as renewal and differentiation (Caires *et al.*, 2012).

Vascular Endothelial Growth Factor A \((Vegfa)\) is a complex gene comprised of eight exons and seven introns that can be alternatively spliced into various isoforms, both pro- and antiangiogenic (Harper and Bates, 2008). VEGFA operates through two primary
receptors- kinase insert domain receptor (KDR or VEGFR2) and fms-like tyrosine kinase (FLT1 or VEGFR1) as well as a co-receptor that augments signal transduction through KDR, neuropilin-1 (NRP-1; Yamada et al., 2001). NRP-1 can only bind proangiogenic isoforms. It can stabilize existing VEGFA and KDR heterodimer complexes or facilitate VEGFA binding to KDR (Pan et al., 2007). VEGFA does not require NRP-1 to signal through KDR; however, its signal transduction is more robust with the co-receptor bound (Soker et al., 1998).

In vivo treatment of male mice (P3-P5) with VEGFA164, 165b or VEGFA165b antibody resulted in novel effects of VEGFA on undifferentiated spermatogonia. Germ cell transplantation for 8 weeks following VEGFA164 and 165b treatment in neonatal mice demonstrated that VEGFA165b treatment reduced the number of SSC colonies in recipients as well as reduction of colony length when compared to either control or VEGFA164 treatments of the donors collected at P22, a time point that might be more suggestive of long-term effects (Caires et al., 2012). The implications of these findings are that VEGFA164 promotes self-renewal of undifferentiated spermatogonia whereas VEGFA165b stimulates differentiation (Fig. 1).

To further identify the role of VEGFA isoforms on the spermatogonial stem cell niche, testis morphogenesis and male fertility, we developed a floxed Vegfa mouse line mated to a pDmrt1-cre line to reduce the expression of VEGFA isoforms in Sertoli and germ cells (pDmrt1-cre;Vegfa^-/-). Testis, epididymis and prostate weights were reduced as well as the number of sperm in the caput epididymis. Furthermore, expression of genes involved in apoptosis/survival, and expression of genes and proteins important for undifferentiated spermatogonia development were all increased or tended to be increased
in \textit{pDmrt1-cre;Vegfa}^{-/} compared to controls. Some of the results were surprising since dramatic reductions in all VEGFA isoforms (both proangiogenic and antiangiogenic) caused increases in genes regulating the stem cell niche and increases in genes that regulate apoptosis while in contrast spermatogenesis was reduced. These increases in gene expression may have been over-compensation of the testis in response to limited or no expression of all VEGFA isoforms. Additionally in this mouse line male fertility was compromised with increased numbers of days between first mating and first parturition and increased number of days between first parturition and second parturition in both knockout mice and heterozygous mice. Thus, we surmised that VEGFA isoforms impact fertility via an affect on spermatogenesis, undifferentiated spermatogonia renewal and survival.

Therefore, for the current study, we sought to investigate the mechanisms of VEGFA isoforms on the spermatogonial stem cell niche by inducing an imbalance in VEGFA isoforms \textit{in vivo} by reducing VEGFA angiogenic isoform activity. Specifically, we reduced the activity of VEGFA angiogenic isoforms by knocking down the NRP-1 co-receptor in Sertoli cells. Since NRP-1 cannot bind antiangiogenic isoforms of VEGFA, this would adversely impact proangiogenic VEGFA signaling resulting in a relative increase in activity of antiangiogenic VEGFA isoforms. We hypothesized that this imbalance of actions of VEGFA angiogenic to antiangiogenic isoforms would impair testis development, cause apoptosis in different stages of spermatogenesis and stimulate differentiation rather than self-renewal in undifferentiated spermatogonia. These experiments could also elucidate a role for NRP-1 in SSC homeostasis.
Materials and Methods

Animals

Sertoli cell-specific NRP-1 knockout mice were generated via the Cre recombinase-loxP system. A C57B6 background, floxed Nrp-1 mouse line was obtained from Jackson Labs (Bar Harbor, ME). The Nrp-1 mice were mated with an Amhr2-cre line (Mutant Mouse Regional Resource Center, MMRRC; Chapel Hill, NC) to obtain male mice with Sertoli and reproductive tract-cell-specific Nrp-1 knockouts (See Appendix A for breeding scheme). Amhr2 is expressed at 12.5 dpc in both sexes, and expression is localized to Sertoli cells in males and correlates with the onset of spermatogenesis (Arango et al., 2008). Immunohistochemistry (Fig. 11) was used to confirm the knockout. Amhr2-Cre;Nrp-1−/− knockout mice (n=5) were null for Nrp-1 and cre-positive. Amhr2-Cre;Nrp-1+/− males (n=11) were homozygous for Nrp-1 and cre-negative; this nomenclature was assigned to controls. Males were collected for blood for hormone ELISA, and testes, epididymides, prostates, seminal vesicles, kidneys and adrenal weights were measured. The mice averaged 78.6 d of age at the time of collection. All mice were housed in a viral-free facility at the University of Nebraska-Lincoln (UNL). Animal procedures were all approved by the UNL Institutional Animal Care and Use Committee (IACUC).

Genotyping

DNA was extracted from tail segments taken from the mice and genotyped via PCR. Proteinase K digestion and 6M NaCl extraction and ethanol precipitation were used to extract the genomic DNA (Bott et al., 2010). After RNA was extracted from testes and converted to cDNA conventional PCR was also used to determine gonadal cre
expression. Males were removed from the study if gonadal cre differed from the previously determined genomic cre regardless of Nrp-1 allelic expression.

**Fixation, Embedding, and Staining**

Testes and epididymides were fixed in Bouin’s solution for 1 h and then rinsed in 70% ethyl alcohol in water (EtOH) until the solution was no longer yellow. Tissues were then, rinsed for 1 h in each of three 100% EtOH baths and for 1 h in each of three baths of CitriSolv (Fisher Scientific; Fairlawn, IL). Rehydrated testes and epididymides were left in liquid paraaffin overnight and embedded in paraaffin the following morning. The embedded tissues were sliced into 10 slides of at least 6 5 μm sections each to ensure the availability of middle sections. Sectioning was also performed on the epididymis. Slides were deparaffinized, rehydrated and stained with hematoxylin (VWR International; Westchester, PA) and eosin (Ricca; Arlington, TX) (H&E) for morphology according to a previously adapted protocol.

**Immunohistochemistry**

Middle sections of testes were used for immunohistochemical analysis. Slides were deparaffinized, rehydrated and microwaved for 15 min to boil in 0.01 M sodium citrate for antigen retrieval. Slides were cooled for 1 h at room temperature and then rinsed in 3% hydrogen peroxide (H₂O₂) in methanol for 20 min and then in 1x PBS 3 times for 2 min each. A Pap pen (Research Products International Corp.; Mount Prospect, IL) was used to isolate a negative control section and treatment section. Sections were blocked with 10% normal goat serum (NGS; PAA Laboratories, Inc.; Ontario, Canada) diluted in PBS for 30 min. NGS was left on the negative control section to prevent drying out but removed from treatment sections. A 1:500 dilution of a PLZF (rabbit polyclonal
Ab; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA; Catalog #sc-22839) primary antibody diluted in PBS was left on the treatment sections overnight while the slides were covered and stored at 4°C. Next, slides were rinsed 3x for 2 min in PBS. A secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Inc.; Burlingame, CA) diluted in PBS at 1:300 was placed on all sections while slides incubated for 30 min. Two drops of Vectastain ABC Kit (Goat IgG; Vector Laboratories, Inc.; Burlingame, CA) in PBS were added to each section after another rinsing in 3 PBS washes for 2 min each; this was 20 min incubation. Vector NovaRED Substrate Kit (Vector Laboratories, Inc.; Burlingame, CA) was added to sections following another 3x 2 min PBS rinse. The solution was left on for 1 min, and slides were placed in distilled water to halt the reaction. Slides were blotted between all stages after standing in the distilled water; they were cover-slipped after the addition of mounting media (Abcam; San Francisco, CA). Stained and covered slides were covered and stored at 4°C. The same protocol was used for NRP-1 expression but with a NRP-1 antibody (rabbit polyclonal Ab; ECM Biosciences; Versailles, KY; Catalog #NP2111) diluted 1:250 in PBS.

**Imaging and Testicular and Epididymal Cell Counts**

Three or four 40x images of each testis and each epididymis slide to be counted were taken via an Olympus DP71 camera and Olympus BX51 microscope; the software used was cellSens Standard. Images were also taken at 20x magnification of testes. Red brown staining represented either PLZF or NRP-1 expression in the testes. The number of PLZF-positive, NRP-1-positive or sperm cells for each image per animal were counted by two technicians and averaged in animal and again within genotype. Cell counts were recorded for statistical analysis.
**TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) Assay**

Middle sections of control and knockout testes were assayed for apoptosis according to a previously devised set of instructions (Promega; Madison, WI; Catalog #G3250). Slides were mounted using a VectaStain mount with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.; Burlingame CA). Testes were imaged in a dark room using SlideBook software and using an Olympus Hamamatsu ORCA-ER microscope and camera. Apoptosis-positive cells fluoresced bright green (FITC filter) while nuclei shone bright blue because of the DAPI. Three images were taken at 10x for counting by two technicians and two images taken at 40x for potential comparison. Cells that expressed apoptosis were counted per tubule.

**RNA Extraction and Quantification**

One testis was collected from each mouse for RNA extraction, reverse transcription to cDNA and QPCR analysis. Testes were stored in Tri-Reagent (Sigma; St. Louis, MO) and homogenized using a 1 CC syringe and increasing gauge needles as the tissue surface area increased. Subsequent RNA extraction began at room temperature with adding 0.2 mL of chloroform to each sample, vortexing for 15 sec and letting them set for 5 min. Samples were then centrifuged at 4°C for 10 min at 12,000 g. Once the supernatants were removed and transferred to new microcentrifuge tubes, 0.5 mL isopropyl alcohol was added to each sample. Samples were vortexed and allowed to sit at room temperature for 5 min; they were again centrifuged for 10 min at 12,000 g at 4°C. 1 mL 75% EtOH in 25% diethyl-pyrocarbonate (DEPC)-treated water was added. Samples were vortexed and centrifuged at 4°C for 5 min at 12,500 g. Supernatants were poured off; samples were allowed to air dry; and 20 μL of DEPC water were added to each
ethanol pellet. 1 μL was added to DEPC water for a 1:14 dilution. This solution was used to quantify the concentration of RNA (ng/mL) present via a Lambda EZ 150 Nano Drop Spectrophotometer (Perkin Elmer). Absorbencies at both 260 nm and 280 nm were measured, and a ratio of the two was calculated to determine concentration of RNA. Samples were stored at -80°C until reverse transcription was performed.

Reverse Transcription to cDNA

DEPC water was added to enough concentrated RNA to reach a 15 μL reaction mixture for each sample and to result in 5 μg of cDNA. A master mix was added to each sample consisting of 5 μL DNase (Promega; Madison, WI; Catalog #M6101) and 5 μL 5x First Strand Buffer (Invitrogen) to eliminate any DNA contamination. Samples were incubated in a PTC-200 thermal cycler (MJ Research; Quebec, Canada) for 30 min at 37°C. 1 μL DNase Stop Solution (Promega) was added to each sample, and samples were incubated at 65°C for 10 min. To conclude the DNase treatment reaction, 1 μL each sample was pipetted into a new tube with 15 μL DEPC water to represent ‘No Reverse Transcription (No RT)’ controls. These samples were pooled and demonstrated whether there was residual genomic DNA when Real-Time PCR was performed; Gapdh was the sole QPCR for the No RTs. A master mix comprised of 2 μL DEPC water, 1 μL Random Primers (Promega; Catalog #C118A), and 1 μL 10 mM deoxynucleotide triphosphates (dNTPs; Promega; U151B) was added. Samples were incubated for 5 min at 65°C and then chilled on ice. Subsequently, a second master mix was added with 4 μL 5x First Strand Buffer (Invitrogen Life Technologies; Carlsbad, CA), 1 μL 0.1 M DL-dithiothreitol (DDT; Invitrogen), 1 μL RNaseOUT (Invitrogen), and 1 μL of a reverse transcriptase, Super Script III (Invitrogen). Each sample was briefly centrifuged. Samples
were incubated at 50°C for 50 min, 70°C for 5 minutes and again chilled on ice. They were briefly centrifuged and had 1 μL RNase H (Invitrogen) added to further eliminate RNA contamination. After a 20 min incubation at 37°C, samples were stored at -20°C until used for QPCR.

*Real-Time PCR (QPCR)*

Master mixes were added to 0.5 μL cDNA template in Quantitative Real-Time PCR (QPCR). Taqman Universal PCR and Power SYBR Green Master Mixes were used (Applied Biosystems; Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was the housekeeping gene to which all others were normalized as its expression is constitutive; rodent *Gapdh* primers and probe came from a predesigned kit. See Table 1 for kits and sequences for genes in this study. QPCR was performed using a 384-well plate, and samples were run in triplicate in separate consecutive wells. The machine (7900, Applied Biosystems) cycled through different stages- 50.0°C for 2 min, 95°C for 10 min, 95°C for 15 sec and then 60°C for 1 min (repeated for as many cycles as the user sets), and finally 95°C for 15 sec. Different dilutions of cDNA from similar tissues as the unknowns were used as standards. An arbitrary value was given to the undiluted standard, and values were ascribed to subsequent dilutions. Those values were plotted against the cycle threshold value where amplification is first detected to synthesize the standard curve. Taqman Master Mix was used for *Gapdh, Bcl2, Bax, Sin3a, Gdnf, Ret* and *Neurog3*. In addition to the 1 μL template, master mixes included 0.5 μL each of 20 μM forward and reverse primers, 2 μL 2 μM probe, 10 μL 2x TaqMan and 6 μL Millipore water. Genes that were measured via a TaqMan Gene Expression Assay (Applied Biosystems) required 10 μL TaqMan Master Mix, 8 μL Millipore water, and 1 μL of the
all-inclusive probe and primer kit. SYBR Green was used for *Nanos2*, *Bcl6b*, *Gfra1*, *Plzf*, *Kitl*, *c-Kit*, *Redd1* and *mTorc1*. Primers for use with SYBR Green were designed with Primer Express 3.0 software (Applied Biosystems) and synthesized at Integrated DNA Technologies, Inc. (Coralville, IA). Those reactions required 10 μL of Power SYBR Green, 0.6 μL each of 20 μM forward and reverse primers, and 7.8 μL Millipore water. Genes that required SYBR to measure were also plotted on a dissociation curve to ensure that there was not primer dimerization due to the lack of a probe. The dissociation curve added a new stage for the machine to cycle through- 95°C for 15 sec, 60°C for 15 sec and then finished again at 95°C for 15 sec. Unknown values were plotted along the standard curve. Average quantities for unknowns were divided by average quantities of *Gapdh* for the same samples to derive relative quantities. All individual quantities were divided by the means for controls which resulted in the controls equaling ‘one’ and treatments being represented as fold changes. Data were then analyzed statistically.

**Blood Collection and Hormone Quantification**

Trunk blood was collected at the time of euthanasia and placed in microcentrifuge tubes containing 20 μL 30% potassium EDTA (Fisher Scientific Co.; Fair Lawn, NJ). Blood was centrifuged immediately after collection for 12,000 g at 4°C for 10 min. Testosterone concentration was determined by an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Alpha Diagnostics International, Inc.; San Antonio, TX; Catalog #1880). Quantities were determined by the manufacturer’s protocol. The standard curve consisted of samples with concentrations of 0 ng/mL, 1 ng/mL, 2.5 ng/mL, 10 ng/mL and 20 ng/mL. The sensitivity of the ELISA was 0.125 ng/mL. Data were transformed using Prism and were plotted against a curve derived from provided standards.
**Statistical Analysis**

All data were analyzed in JMP, a business unit of SAS (SAS Institute; Cary, NC). The Dunnett’s procedure was used to compare Amhr2-cre;Nrp-1−/− values to those of controls. A t-test was used when heterozygous animals were incorporated in order to compare all pairs. Differences were considered significant at P<0.05. Data were considered to be approaching significance if P<0.1 but P>0.05.

**Results**

**NRP-1 Immunohistochemistry**

While no differences were seen in the gross morphology of the testes of Amhr2-Cre;Nrp-1−/− mice (Fig. 2B and D) compared to controls (Fig. 2A and C), there were differences in the expression of protein for NRP-1 with dramatic reductions to no expression in Sertoli. Furthermore, there was reduced expression of NRP-1 even in the number of germ cells per tubule (30.056±10.69 cells vs 100.58±15.11 cells, P < 0.02; Fig. 2E).

**Amhr2-Cre;Nrp-1−/− Male Organ Weights and Testosterone**

There was an increase in the weights of Amhr2-Cre;Nrp-1−/− testes compared to controls (0.2±0.0055 g vs 0.17±0.004 g, P < 0.003; Fig. 3A). The prostates of Amhr2-Cre;Nrp-1−/− males were also increased by 1.98-fold (0.042±0.009 g vs 0.021±0.0039 g, P < 0.03; Fig. 3C).

No differences were observed in the weights of the epididymides (Fig. 3B) or of the seminal vesicles (Fig. 3D). Additionally, kidney and adrenal weights (Fig. 3E-F) were not different. Finally, total body weight did not differ between Amhr2-Cre;Nrp-1−/− male mice and controls (Fig. 3G).
In addition to weighing various organs at the time of collection, serum testosterone was measured via ELISA. Testosterone was not significantly different between Amhr2-Cre;Nrp-1⁻/⁻ males and control males (Fig. 3).

**QPCR of Undifferentiated Spermatogonia-related Genes in Sertoli Cells**

As seen in Figure 4, we looked at genes expressed in Sertoli cells of the testis. Amhr2-Cre;Nrp-1⁻/⁻ testes had a significant 6.58-fold reduction in Sin3a mRNA compared to controls (0.15±0.06 vs 1±0.24, P < 0.04; Fig. 4A). Knockout males tended to have less Kitl expression than controls (0.24±0.098 vs 1±0.26, P < 0.1; Fig. 4B).

**QPCR of Gdnf and Its Co-receptor and Receptor**

Gdnf is another Sertoli cell-produced factor, and it is one of few factors known to be necessary for undifferentiated spermatogonia self-renewal (Meng *et al.*, 2000). Its receptor, Ret, and co-receptor, Gfra1, are expressed in undifferentiated spermatogonia (Suzuki *et al.*, 2009). Gdnf mRNA was significantly reduced in knockouts in comparison to controls (14.75-fold; 0.068±0.037 vs 1±0.44, P < 0.05; Fig. 5A). Ret mRNA tended to be reduced 2-fold in Amhr2-Cre;Nrp-1⁻/⁻ testes compared to controls (0.5±.18 vs 1±0.16, P < 0.09; Fig. 5B). However, there were no differences in either Gfra1 expression (Fig. 5C).

**QPCR of Genes Expressed by Spermatogonia**

We examined the mRNA abundance of genes expressed in both undifferentiated and differentiated spermatogonia. Neurog3, a marker in undifferentiated spermatogonia thought to possibly be an early marker of differentiation (Yoshida *et al.*, 2004), was significantly diminished in Amhr2-Cre;Nrp-1⁻/⁻ testes relative to controls (7.9-fold, 0.13±0.049 vs 1±0.16, P < 0.01; Fig. 6A). Figure 6B demonstrates that Plzf expression
was elevated 5.45-fold in knockout testes compared to controls (5.45 ± 0.1 vs 1 ± 0.33, P < 0.002). Plzf is important for self-renewal of undifferentiated spermatogonia (de Rooij et al., 2009). Two other genes important for self-renewal were investigated in this study—Nanos2 and Bcl6b (Suzuki et al., 2009; Oatley et al., 2006, 2007). Neither was different from controls (Fig. 6C-D). A marker for differentiating spermatogonia is c-Kit. In the literature, undifferentiated spermatogonia are considered negative for c-Kit and spermatogonia that stain positively are considered differentiated germ cells (Schrans-Stassen et al., 1999; de Rooij et al., 2009). While the ligand for c-KIT, Kitl, tended to be reduced in knockout males, there were no statistical differences in c-Kit mRNA abundance (Fig. 6E). The final two genes investigated in this study were mTorcl and Redd1. Redd1 serves to inhibit mTorcl activity and is upregulated in response to stressors. Its expression was not different between the testes of Amhr2-Cre;Nrp-1−/− mice and controls (Fig. 6F). mTorcl expression is depicted in Figure 6G; this gene is normally regulated by stress conditions. While it is known for promoting cell growth and proliferation, that comes at the expense of stem cell depletion in some instances (Hobbs et al., 2011; Chen et al., 2008; Brugarolas et al., 2004; Shoshani et al., 2002); its expression was not different.

**PLZF Immunohistochemistry**

With the unanticipated increase in Plzf in Amhr2-Cre;Nrp-1−/− testes, we sought to determine if there was coordinated increases in protein. Figures 7A and 7C show a control testis stained for PLZF at 20x and 40x, respectively; 7B and 7D represent a knockout testis at the same magnifications. Figure 7E demonstrates that the number of
PLZF-expressing cells per tubule was, indeed, elevated in knockout males (85.23±11.94 cells vs 22.10±4.26 cells, P = 0.0004).

QPCR of Significantly Different Genes Including Heterozygous Animals

Genes mentioned previously are still significantly different or tending to be different when heterozygous animals are included. *Amhr2-cre;Nrp-1*+/− males were intermediate between controls and knockouts for *Ret* but also interestingly for both Sertoli cell-produced genes measured (Sin3a and Kitl). *Sin3a* is shown in panel 8A (0.15±0.06 vs 0.61±0.22 vs 1±0.24, P < 0.04). *Kitl* is seen in Fig. 8C (0.24±0.098 vs 0.53±0.19 vs 1±0.26, P = 0.06). The *Amhr2-cre;Nrp-1*+/− males had Gdnf values that were similar to the *Amhr2-cre;Nrp-1*−/− mice (0.068±0.037 vs 0.10±0.035 vs 1±0.44, P < 0.07; Fig. 8E). It would seem expressing one floxed allele of *Nrp-1* may result in a dramatic effect and especially for the Sertoli cell factors. *Ret* is depicted in Fig. 8F (0.5±.18 vs 0.72±0.17 vs 1±0.16, P < 0.08). Aside from *Ret* mRNA, the other significantly different genes expressed by undifferentiated spermatogonia appeared to require both floxed alleles to induce a change- *Neurog3* (0.13±0.049 vs 1.42±0.46 vs 1±0.16, P < 0.02; Fig. 8B) and *Plzf* (5.45±0.1 vs 1.15±0.46 vs 1±0.33, P < 0.002; Fig. 8D). Based on these data, NRP-1 inactivation may have an additive effect on gene expression.

Expression of Survival and Apoptosis Genes in Testes

*Bcl2* is a pro-survival gene while *Bax* is pro-apoptotic. In the testes of *Amhr2-Cre;Nrp-1*−/− males, *Bcl2* was significantly reduced by 4.43-fold in knockouts (0.22±0.055 vs 1±0.18, P < 0.03; Fig. 9A). *Bax* mRNA and expression of *Bcl2* relative to *Bax* were not significantly different (Fig. 9C and E). Expression of both *Casp3* and *Casp9* did not differ between controls and knockouts (Fig. 9B and D).
TUNEL Assay

Because Bcl2 was reduced in the testes of 79-day-old Amhr2-Cre;Nrp-1-/- males, we sought to measure apoptosis using TUNEL. Images in Figure 4 were overlaid with DAPI to represent the nuclei, and bright green fluorescence shows DNA fragmentation. A control testis is shown at 10x (Fig. 10A) and 40x (Fig. 10C). A testis belonging to an Amhr2-Cre;Nrp-1-/- mouse is depicted also at 10x (Fig. 10B) and 40x (Fig. 10D). Cells positive for apoptosis were counted, and the counts are presented graphically in Figure 10E. Occurrence of apoptosis between knockouts and controls was not statistically significant at the 78.6 d age when the testes were collected. There were also no apparent differences in proliferation between controls and Amhr2-Cre;Nrp-1-/- testes by counting cells positive for proliferating cell nuclear antigen (PCNA; Fig. 10F).

QPCR of VEGFA Isoforms

Figure 11 shows the results of QPCR for isoforms of Vegfa. Vegfa164 mRNA expression was not different (Fig. 11A). Messenger RNA abundance of Vegfa165b was also not different (Fig. 11B). Finally, the ratio of proangiogenic Vegfa164 to antiangiogenic Vegfa165b was not different between controls and homozygotes (Fig. 11C).

Sperm Numbers in the Caput Epididymis

Considering differences seen in mRNA abundance and in previous knockout mouse data in our lab (Lu et al., submitted), we sectioned epididymides to determine if differences numbers of spermatozoa existed. Figure 12A shows a control epididymis, and an epididymis taken from a knockout male is presented in 12B. No differences could be
seen between sections, and counting the caput epididymal sperm per tubule confirmed the visual observations (Fig. 12C).

**Fertility Trial**

Two *Amhr2-Cre;Nrp-1*−/− males were placed with control females when they were 82 d old and were allowed to breed until collected at 276 d when they had stopped reproducing. The low probability of producing the *Amhr2-Cre;Nrp-1*−/− genotype (25%) coupled with confirming cre expression in the gonads limited the number of animals. These were compared to 12 pairs in which control males were mated to control females. The number of pups born in the first litter was significantly reduced amongst the knockouts (2±2 pups vs 8.67±0.39 pups, P = 0.0001; Fig. 13A). Averaged numbers of pups born across all litters was reduced when the *Amhr2-Cre;Nrp-1*−/− males were mated to female controls (3.57±1.6 pups vs 8.5±1.53 pups, P = 0.03; Fig. 13B). Figure 13C demonstrates that the average number of days from parturition to subsequent parturition tended to be reduced in the knockout x control matings compared to controls x controls (40.14±4.69 d vs 25.75±6.21 d, P < 0.1). Finally, fewer pups survived after parturition that were born to the knockout by control mating pairs (1.57±0.868 pups vs 8.5±0.5 pups, P = 0.0003; Fig. 13D). These data suggest a form of subfertility when *Nrp-1* is knocked out in Sertoli cells.

**Discussion**

Our study is the first to demonstrate that decreased angiogenic VEGFA isoform actions in Sertoli cells affects genes that regulate the spermatogonial stem cell niche as well as genes that are critical in cell survival. Furthermore, the fertility data is suggestive of a subfertility phenotype with progressive loss of fertility overtime indicative of
accelerated reproductive aging. Utilizing a conditional knockout approach avoided the normal embryonic lethality that occurs when VEGFA or either of its receptors, KDR, FLT1 or NRP-1, is knocked out globally (Ferrara et al., 1996; Kitsukawa et al., 1997). NRP-1 immunohistochemistry of control and Amhr2-Cre;Nrp-1−/− testes revealed that there was dramatic if not complete reduction of Nrp-1 by semi-quantifying subsequent NRP-1 protein expression in Sertoli cells. Strikingly, we noticed a significant reduction of NRP-1 in germ cells as well. We attributed that occurrence to the intimate contact shared from Sertoli cells forming aggregates with germ cells to affect their gene expression as NRP-1 is not a secreted protein (Sharpe, 1994).

We postulated that the knockout of Nrp-1 and the subsequent reduction in proangiogenic VEGFA signal transduction would downregulate genes important for the self-renewal of undifferentiated spermatogonia and would increase apoptosis. While this did occur to some extent, the Cre-loxP system knocked down genes in both Sertoli cells and in germ cells as well as genes important in self-renewal and differentiation. Messenger RNA for Gdnf was reduced in Amhr2-Cre;Nrp-1−/− testes along with its receptor, Ret, that tended to be reduced and is expressed by undifferentiated spermatogonia. As a Sertoli cell-secreted product, GDNF is proposed to be involved in undifferentiated spermatogonial self-renewal similar to proposed functions of PLZF, a protein produced by undifferentiated spermatogonia (Meng et al., 2000; de Rooij et al., 2009). However, neither GDNF nor PLZF have been demonstrated to regulate one another. Expression of Plzf was elevated in testes of knockout males that coincided with the increased PLZF-positive staining suggesting there are more undifferentiated spermatogonia in the knockout testes. Immunohistochemistry for PLZF suggested
increased numbers of undifferentiated spermatogonia because of increased PLZF-positive cells in knockout testes.

In knockout testes, pro-survival Bcl2 was downregulated; however, there was no increased apoptosis via assays for TUNEL or seen via QPCR for either Casp3 or Casp9. Previous data have demonstrated differences in the ratio of Bcl2-to-Bax when angiogenic isoforms of VEGFA were injected (Caires et al., 2009). Additionally, Sin3a, a factor expressed by Sertoli cells that plays a large role in establishment of the niche at the basement membrane of seminiferous tubules, was reduced in knockout testes (Payne et al., 2010).

In addition to finding differences in self-renewal genes and in a factor important for SSC niche establishment, factors important for differentiation of undifferentiated spermatogonia were also significantly different in testes of Amhr2-cre;Nrp-1"- males. Neurog3 is a marker of differentiation that is expressed in undifferentiated spermatogonia that ultimately give rise to spermatogenesis (Yoshida et al., 2004), and it was reduced in this study. While mRNA for the known differentiation marker c-Kit was not different between knockouts and controls, Kitl expression tended to be reduced in Amhr2-Cre;Nrp-1"- testes. This ligand to c-Kit is important for gonocyte migration and survival, but its secreted form maintains proliferation of differentiated, c-KIT-positive spermatogonia (Manova et al., 1993; de Rooij et al., 1999; Ohta et al., 2000; Ohta et al., 2003; Laird et al., 2011).

A form of subfertility was also seen resulting from Amhr2-Cre;Nrp-1"- males. Fewer pups were born to the first litter and then over all litters. Fewer pups survived to weaning, and the length between parturitions tended to increase over time. Mice were
mated at approximately 60 d and allowed to breed to cessation. While we still need to assay messenger RNA abundance and protein expression in the aged tissue, the reduction in fertility is presumably due to a progressive loss of sperm over time.

It has been demonstrated that the balance of proangiogenic isoforms of VEGFA to antiangiogenic is important as a result of spermatogonial stem cell analysis through germ cell transplantation assays, the sole means to determining if the SSC population has been affected. We also have determined that reduction of all VEGFA isoforms, both proangiogenic and antiangiogenic, in Sertoli and germ cells affects testis morphogenesis, expression of genes that regulate the stem cell niche, reduces the number of undifferentiated spermatogonia and results in subfertility. Our current study with the floxed Nrp-1 line further supports the effects of VEGFA isoforms on the process of undifferentiated spermatogonial renewal, differentiation and survival and also appears to have a subfertility phenotype as well as that an imbalance of VEGFA isoforms in vivo yields lasting effects. Thus, our lab has devised a model of how VEGFA164 and VEGFA165b could be involved to equilibrate self-renewal and homeostasis (Figure 14).

VEGFA164 upregulates GDNF and also increases RET phosphorylation in the kidney (Tufro et al., 2007). Considering our Sertoli cell knockout would eliminate Nrp-1 expression and would pose a major hindrance to VEGFA164 signaling, we suspect that VEGFA164 functions similarly on GDNF and RET in the testis. Sertoli cells produce GDNF, and it binds a heterodimer of RET and GFRA1 in order to carry out self-renewal (Suzuki et al., 2009); however, it also upregulates other self-renewal factors expressed in undifferentiated spermatogonia- BCL6B and NANOS2 (Oatley et al., 2006, 2007; Sada et al., 2012). In embryonic pancreata, GDNF has been shown to increase NEUROG3
expression, a factor that is an early marker for differentiation expressed in A_al spermatogonia (Gasa et al., 2004). This would suggest that, as a major inductor of self-renewal, GDNF also recruits factors for differentiation to keep the two processes balanced. These suggestions are backed by seeing reductions in both Gdnf and Neurog3 gene expression in the knockout testes compared to controls in our study. While there was a decrease in Gdnf mRNA abundance and a tendency for decreased Ret expression, Plzf was elevated. It is one of the other major factors that promote self-renewal and is not regulated by GDNF. However, PLZF can indirectly affect RET by upregulating REDD1, and REDD1 will block the ability of mTORC1 to inhibit RET function (Hobbs et al., 2011). Since Redd1 and mTorc1 mRNA abundance did not differ in this study, we suspect the tendency for reduced Ret to be a direct result of the imbalance of VEGFA.

PLZF is proposed to stimulate renewal by repression of c-KIT, a major marker of differentiation in spermatogonia (Filipponi et al., 2007). Considering the evidence that factors known for renewal can stimulate differentiation downstream as well, we suggest that VEGFA165b upregulates PLZF to maintain the balance between self-renewal and differentiation. This could be in addition to or instead of the idea that VEGFA164 downregulates PLZF since it normally stimulates GDNF. While there were no differences in c-Kit expression in testes, its Sertoli cell-based ligand, Kitl, tended to be reduced in knockouts. Kitl is important for early gonocyte migration and cell proliferation (Manova et al., 1993; Deshpande et al., 2010). A study by Heissig and others (2005) demonstrated that peritoneal addition of recombinant VEGF in mice increased plasma concentrations of KITL in mice in vivo. If this effects holds true for the testis, our recent data would suggest that KITL is downregulated either by increased action of VEGFA165b or by
reduced activity by VEGFA164. Finally, Sin3a was reduced in knockout testes, as well. Although regulation of SIN3A is largely unknown, it could be that VEGFA or NRP-1 stimulation is important for maintaining the spermatogonial stem cell niche environment.

Our lab is the first to conditionally knock out NRP-1 in Sertoli cells and conclude that NRP-1 activity greatly impacts the ability of VEGFA proangiogenic isoforms to promote development of undifferentiated spermatogonia. These data mirror the findings of Caires and others (2012) that VEGFA164 promotes self-renewal and that VEGFA165b drives differentiation. However, in addition to further implicating a role of VEGFA in SSC development, this study sheds light on the impact NRP-1, the co-receptor to proangiogenic isoforms of VEGFA, on SSC homeostasis. Considering the expression patterns of NRP-1 correlating with undifferentiated spermatogonia renewal (Caires et al., 2012), NRP-1 may be a prime candidate as a novel stem cell marker.
Figure Legends

Table 1. Gene expression assay kits, primer and probe sequences and vendors.

Figure 1. Proposed effects of VEGFA isoforms on differentiation and renewal of undifferentiated spermatogonia including the genes from this study thought to be involved.

Figure 2. NRP-1 immunohistochemistry and counts of NRP-1-positive cells per tubule. A) controls testis at 20x, B) Amhr2-cre;Nrp-1+/− testis at 20x, C) Controls testis at 40x, D) Amhr2-cre;Nrp-1+/− testis at 40x, and E) graph of average total NRP-1-positive cells per image. Significance was set at P < 0.05. For controls, n = 4; for Amhr2-cre;Nrp-1+/−, n = 3.

Figure 3. Effects of NRP-1 knockout on male organ weights and serum testosterone. A) Testis weight, B) Epididymides weight, C) Prostate weight, D) Seminal Vesicles weight, E) Kidneys weight, F) Adrenals weight, G) Total Body Weight, and H) Testosterone. Significance was set at P < 0.05. For controls, n = 11; for Amhr2-cre;Nrp-1+/−, n = 5.

Figure 4. mRNA abundance of Sertoli cell-expressed genes. A) Sin3a and B) Kitl. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P<0.05. Data were considered tending toward significance when 0.1 > P > 0.05. For controls, n = 10; and for Amhr2-cre;Nrp-1+/− , n = 4.

Figure 5. Sertoli cell-derived Gdnf and its germ cell-expressed co-receptor components expression. A) Gdnf, B) Ret and C) Gfra1. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P < 0.05. For controls, n ≥ 6; and for Amhr2-cre;Nrp-1+/−, n = 4.

Figure 6. Expression of genes expressed in germ cells. A) Neurog3, B) Plzf, C) Nanos2, D) Bcl6b, E) c-Kit, F) Redd1 and G) mTorcl. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P < 0.05. For controls, n ≥ 8; and for Amhr2-cre;Nrp-1+/−, n ≥ 3.

Figure 7. PLZF immunohistochemistry and counts of PLZF-positive undifferentiated spermatogonia per tubule. A) Control testis at 20x, B) Amhr2-cre;Nrp-1+/− testis at 20x, C) Control testis at 40x, D) Amhr2-cre;Nrp-1+/− testis at 40x, and E) graph of average total PLZF-positive cells per image. Significance was set at P < 0.05. For controls, n = 6; for Amhr2-cre;Nrp-1+/−, n = 4.

Figure 8. Statistically significant QPCR data including Amhr2-cre;Nrp-1+/− (heterozygote) testes. Sertoli-cell genes are depicted on the left- A) Sin3a, C) Kitl, E) Gdnf. Genes expressed by undifferentiated spermatogonia are shown on the right- B)
Neurog3 and D) Plzf. Significance was set at P<0.05. Data were considered tending toward significance when 0.1 > P > 0.05. For controls, n ≥ 5; for Amhr2-cre;Nrp-1<sup>1−/−</sup>, n ≥ 5; and for Amhr2-cre;Nrp-1<sup>−/−</sup>, n = 4.

**Figure 9.** QPCR of genes important for survival and apoptosis. A) Bcl2, C) Bax, E) Bcl2:Bax, B) Casp3, D) Casp9. Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. Significance was set at P < 0.05. For controls, n = 10; for Amhr2-cre;Nrp-1<sup>−/−</sup>, n = 4.

**Figure 10.** TUNEL assay images and apoptosis-positive cell counts per tubule. Green fluorescence denotes cells that are positive for apoptosis. Blue staining is indicative of DAPI staining in cell nuclei. Control testes are represented by images A and C. Amhr2-cre;Nrp-1<sup>−/−</sup> testes are shown in panels B and D. 4E shows the graph of apoptotic cells in both controls and knockouts. PCNA-positive cells per tubule are shown in panel 4F. For controls, n ≥ 3; for Amhr2-cre;Nrp-1<sup>−/−</sup>, n = 3.

**Figure 11.** Expression of proangiogenic Vegfa164 (A), antiangiogenic Vegfa165b (B), and the ratio, Vegfa164:Vegfa165b (C). Graphs were represented as fold changes compared to controls set at one. All samples were normalized to Gapdh. For controls, n ≥ 6; and for Amhr2-cre;Nrp-1<sup>−/−</sup>, n ≥ 1.

**Figure 12.** Epididymal histology and caput epididymis sperm counts per tubule. A) Control epididymis at 40x, B) Amhr2-cre;Nrp-1<sup>−/−</sup> epididymis at 40x and C) graph of average number of spermaotozoa per image in the caput epididymis. For controls, n = 7; for Amhr2-cre;Nrp-1<sup>−/−</sup>, n = 3.

**Figure 13.** Fertility trial data comparing results from Amhr2-cre;Nrp-1<sup>−/−</sup> males mated to control females (n = 2 litters) with controls mated to each other (n = 12 litters). A) Number of pups born in the first litter, B) Average number of pups in each litter, C) Average number of days between each parturition, D) Average number of pups surviving to weaning. Significance was set at P<0.05. Data were considered tending toward significance when 0.1 > P > 0.05.

**Figure 14.** Proposed model of how VEGFA164 and VEGFA165b interact with SSC factors to promote self-renewal and differentiation, respectively.
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Table 1
Figure 1
Figure 2
Figure 3
Figure 4

A  

Sin3a  
P=0.04

Fold Change  
0  
0.2  
0.4  
0.6  
0.8  
1  
1.2  
1.4

CONTROL  
Amhr2-Cre;Nrp-1/-

B  

Kitl  
P<0.1

Fold Change  
0  
0.2  
0.4  
0.6  
0.8  
1  
1.2  
1.4

CONTROL  
Amhr2-Cre;Nrp-1/-
Figure 5
Figure 6
Figure 7
**Figure 8**

A. Sin3a

B. Neurog3

C. Kitl

D. Plzf

E. Gdnf

F. Ret

Fold Change

CONTROL  Amhr2-Cre;Nrp-1+/+  Amhr2-Cre;Nrp-1/+-

Fold Change  CONTROL  Amhr2-Cre;Nrp-1+/+  Amhr2-Cre;Nrp-1/+-

Fold Change  CONTROL  Amhr2-Cre;Nrp-1+/+  Amhr2-Cre;Nrp-1/+-

Fold Change  CONTROL  Amhr2-Cre;Nrp-1+/+  Amhr2-Cre;Nrp-1/+-

Fold Change  CONTROL  Amhr2-Cre;Nrp-1+/+  Amhr2-Cre;Nrp-1/+-

Legend:

- a: Significant difference compared to CONTROL
- b: Significant difference compared to Amhr2-Cre;Nrp-1+/+

Statistical Significance:

- Sin3a: P<0.04
- Neurog3: P<0.02
- Kitl: P=0.06
- Plzf: P<0.002
- Gdnf: P<0.07
- Ret: P=0.08
Figure 9
Figure 10
Figure 11
Figure 12
Figure 13
Figure 14
Literature Cited


Summary

Our hypothesis was that skewing the balance of VEGFA isoforms through knocking out Nrp-1 in mouse Sertoli cells or by injection in perinatal rats would result in an inability of pro-angiogenic VEGFA isoforms to signal as efficiently; and thus reduce their actions. VEGFA has been shown to upregulate GDNF and to increase RET phosphorylation in the kidney; thus, we believe that VEGFA164 elicits direct effects upon GDNF and its ability to induce self-renewal of undifferentiated spermatogonia (Tufro et al., 2007). Furthermore, the balance of VEGFA angiogenic to antiangiogenic isoforms may affect expression of GDNF.

In contrast to a reduction of Gdnf in the Nrp-1 knockouts, we witnessed a drastic increase both in Plzf mRNA and PLZF protein expression, a transcription factor that is important for self-renewal and germline maintenance and has been shown not to be regulated in any way by GDNF in the rat (Schmidt et al., 2009). However, PLZF does indirectly affect RET expression. Normally, PLZF can stimulate expression of REDD1 (also known as DDIT4, RTP801, or DIG2) in the germ cells; REDD1 is a stress-induced factor that inhibits activity of mammalian target of rapamycin complex 1 (mTORC1). The function of mTORC1 is to augment translation and cell growth and is regulated by such stimuli as growth factors and cell stress. While its increase leads to higher proliferation, mTORC1 also depletes stem cell reservoirs in certain tissues. It was demonstrated that introducing an mTORC1 inhibitor to Plzf−/− undifferentiated spermatogonia in culture resulted in low levels of Ret mRNA and Gfra1 mRNA returning to basal levels (Hobbs et al., 2011; Chen et al., 2008; Brugarolas et al., 2004; Shoshani et al., 2002). Essentially, PLZF recruits REDD1 to block the inhibition of mTORC1 on RET activity.
This indirect action of PLZF on RET may serve to further keep up self-renewal; however, it could be a direct result of VEGFA. Since we saw a tendency for reduced Kitl mRNA in knockout testes when Nrp-1 was knocked out and proangiogenic VEGFA inactivated, we speculate that lesser signaling by VEGFA164 or increased relative effects of VEGFA165b are affecting Kitl. As the ligand to c-KIT, KITL is produced by Sertoli cells and functions to promote cell survival and to maintain differentiated spermatogonia (Manova et al., 1993; Deshpande et al., 2010). A study by Heissig and others (2005) demonstrated increased plasma KITL following IP addition of VEGF. Additionally, inactivation of proangiogenic isoforms of VEGFA would result in a relative increase in the activity of endogenous antiangiogenic isoforms (VEGFA165b). Novel experiments incorporating germ cell transplantation suggest that VEGFA165b drives undifferentiated spermatogonia to progress to later stages of development (Caires et al., 2012). Thus, we speculate that PLZF is upregulated in response to the increased VEGFA165b activity to equilibrate renewal and differentiation.

While the mouse study has the potential to demonstrate long-term effects, SSC genes were affected by introducing antiVEGFAxxxb, an antibody to all antiangiogenic isoforms, to perinatal rats in vivo. Not only was Ret tending to be reduced, but Nanos2, a factor important for renewal and stemness, was significantly reduced. Thus, Figure 1 summarizes all interactions of genes tending to be different. Ultimately, messenger RNA abundance differed as a result of shifting the balance of VEGFA proangiogenic to antiangiogenic isoforms.
Figure 1. The balance of proangiogenic isoforms of VEGFA (164) and antiangiogenic VEGFA (165b) is necessary for appropriate renewal of stem cells and differentiation to maintain spermatogenesis. This model proposes the balance and interactions of VEGFA164 and 165b with undifferentiated spermatogonia niche factors, renewal factors and differentiation factors.