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VEGFA: Just one of multiple mechanisms for Sex-Specific Vascular Development within the testis?

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Abstract

Testis development from an indifferent gonad is a critical step in embryogenesis. A hallmark of testis differentiation is sex-specific vascularization which occurs as endothelial cells migrate from the adjacent mesonephros into the testis to surround Sertoli-germ cell aggregates and induce seminiferous cord formation. Many *in vitro* experiments have demonstrated that Vascular Endothelial Growth Factor A (VEGFA) is a critical regulator of this process. Both inhibitors to VEGFA signal transduction and excess VEGFA isoforms in testis organ cultures impaired vascular development and seminiferous cord formation. However, *in vivo* models using mice which selectively eliminated all VEGFA isoforms: in Sertoli and germ cells (*pDmrt1-Cre;Vegfa*$^{-/-}$); Sertoli and Leydig cells (*Amhr2-Cre;Vegfa*$^{-/-}$) or Sertoli cells (*Amh-Cre;Vegfa*$^{-/-}$ and *Sry-Cre;Vegfa*$^{-/-}$) displayed testes with observably normal cords and vasculature at postnatal day 0 and onwards. Embryonic testis development may be delayed in these mice; however, the postnatal data indicate that VEGFA isoforms secreted from Sertoli, Leydig or germ cells are not required for testis morphogenesis within the mouse. A *Vegfa* signal transduction array was employed on postnatal testes from *Sry-Cre;Vegfa*$^{-/-}$ versus controls. *Ptgs1* (*Cox1*) was the only upregulated gene (5-fold). COX1 stimulates angiogenesis and upregulates, VEGFA, Prostaglandin E2 (PGE2) and PGD2. Thus, other gene pathways may compensate for VEGFA loss, similar to multiple independent mechanisms to maintain SOX9 expression. Multiple independent mechanism that induce vascular development in the testis may contribute to and safeguard the sex-specific vasculature development responsible for inducing seminiferous cord formation, thus, ensuring appropriate testis morphogenesis in the male.

Keywords

VEGFA; testis; sex differentiation; vasculature

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Introduction

Many scientists have been investigating the factor or factors that are responsible for inducing endothelial cell migration from the adjacent mesonephros into the developing indifferent gonad for years. Endothelial cell migration and vascular development has been demonstrated as one of the first steps for directing seminiferous cord formation and initiating the testis differentiation pathway (Albrecht *et al*. 2000; Tilmann and Capel 1999). Within this review we will discuss the reasoning behind investigating VEGFA, its ever evolving complexity due to transcriptional and posttranslational regulation, and how it acts on cells through multiple receptors and co-receptors as indicated mainly by rodent models (Dehghanian *et al*. 2014; Qiu *et al*. 2009; Vieira *et al*. 2010). Since infertility in the human male has been on the rise for almost 50 years (Carlsen *et al*. 1992, 1995; Carlsen *et al*. 1993; Juul *et al*. 2014), more information into gonadal development and how fertility is affected when mutations arise is critical to developing therapies for male related infertility issues. In the last decade, there have also been interesting data to suggest that male infertility may be associated with other health risks since men with subnormal sperm counts also had reduced lifespans when evaluated between 1949 and 1985. This correlation between infertility and shorter lifespans in men may be due to lifestyle and behavior choices, conditions *in utero* or their genetic composition (Groos *et al*. 2006). There have been numerous incidences reported in the scientific literature of prenatal exposure to compounds which alter gene expression through epigenetic mechanisms and may affect reproduction and a host of other critical functions (e.g. immune). Thus, prenatal or *in utero* conditions may be one avenue that can be altered to increase reproductive function in men and also improve their lifelong health status. Since an altered uterine environment may influence gene expression that is necessary for testis development, any additional information on how testes develop may also increase our ability to develop measures to diagnose male infertility disorders that arise through prenatal programming or epigenetic causes.

How does a testis develop from an indifferent gonad?

The Sertoli cell expresses *Sry* (sex determining region of the Y chromosome, a gene that is on the short arm of the Y chromosome) and is the first cell to differentiate in the testis (Magre and Jost 1991). In the mouse, *Sry* is only briefly expressed (E10.5 to 12.5) and its primary function is the upregulation of *Sox9* (SRY-box 9) (Harley *et al*. 2003). Much of the fate of the developing testis appears to be reliant on appropriate Sertoli cell differentiation and on the ability of *Sox9* expression to be maintained at high levels (Figure 1) thereby leading to transcription of many genes to initiate testis development (Harley et al. 2003). Furthermore, expression of *Sox9* upregulates other genes such as Fibroblast Growth Factor 9 (*Fgf9*) which, in addition to cementing the testis differentiation pathway, may antagonize genes such as Wingless type MMTV integration site family, member 4 (*Wnt4*), that if overexpressed or not antagonized will enable development of the female pathway (Jameson *et al*. 2012; Shan *et al*. 2009) (Figure 1).

What is the origin of the Sertoli cell …a cell that is thought to direct testis development?

The precursors of the Sertoli cells are the early somatic progenitor cells which develop from the coelomic epithelium. The coelomic epithelium is derived from the mesonephros and as it differentiates it thickens, a phenomenon proposed to be due to gene expression of *Sry*. Many different transcription factors are critical for the proper development of the gonadal primordium which is enveloped by the coelomic epithelium such as EMX2 (empty spiracles homeobox 2), WT1, NR5A1 (nuclear receptor subfamily 5, group A, member 1, formerly SF1), LHX9 (LIM homeobox protein 9), GATA4 (GATA binding protein 4), ZFPM2/FOG2 (zinc finger protein, multitype 2) and others (Munger and Capel 2012; Wilhelm *et al*. 2007). Both the thickening of the gonadal primordium to form the coelomic epithelium and the expression of *Sry* is critical to allow Sertoli cells to develop. At least 20% of the Sertoli cells need to express *Sry* in order for a testis to arise from the indifferent gonad (Burgoyne *et al*. 1995; Patek *et al*. 1991) (Figure 1). Furthermore, *Sox9* has to be upregulated by E11.2 through the actions of *Sry* for testis development to continue; if *Sox9* is not upregulated then proliferation of the Sertoli cells will arrest along with testis development (Figure 1). In other species such as domestic livestock, *Sry* is maintained much longer and appears to have other functions (Daneau *et al*. 1996; Parma *et al*. 1999; Payen *et al*. 1996; Pelliniemi and Lauteala 1981).

Sox9

Originally *Sox9* is expressed in the indifferent gonad by the pre-Sertoli/granulosa cells and is transcribed at a very low copy number by SF1. When *Sry* is expressed, *Sox9* is upregulated in the testis and its expression is silenced in the ovary (Kobayashi *et al*. 2005). Because *Sry* expression is short-lived, it is critical that other factors continue to upregulate and maintain *Sox9* expression (Figure 1). *Sox9* induces the expression of *Ffg9* and prostaglandin D2 synthase (*Ptgds*), in which the latter generates PGD2 (Wilhelm et al. 2007). One function of both of these genes is to maintain upregulated *Sox9* expression (Rossitto *et al*. 2015). Furthermore, *Sox9* also upregulates itself through two mechanisms. It binds its own enhancer in a feed-forward fashion (Sekido and Lovell-Badge 2008) and by maintaining upregulation of a transcription factor ER71/ETV2 (ets variant 2) which is initially increased through Sry expression (DiTacchio *et al*. 2012).

FGF9

Elimination of FGF9 or its receptor FGFR2 (fibroblast growth factor receptor 2) can cause male-to-female sex reversal given that FGF9 down regulates or suppresses the Wnt pathway which would otherwise promote female gonadal development (Bagheri-Fam *et al*. 2008; Colvin *et al*. 2001; Jameson et al. 2012; Jeays-Ward *et al*. 2003; Siggers *et al*. 2014; Vainio *et al*. 1999) (Figure 1). *Wnt4* knockout mice are sex-reversed similarly to knockouts of FGF9 (Shan et al. 2009). It was determined that FGF9 antagonizes the actions of WNT4 and thus prevents the ovarian pathway and allows for seminiferous cords to develop (Jameson et al. 2012; Kim *et al*. 2006). FGF9 also is critical for Sertoli cell proliferation necessary to provide enough Sertoli cells to differentiate to form clusters with germ cells.

PGD2

The gene encoding *Ptgds* (Lipocalin-type prostaglandin D2 synthase), an enzyme that produces PGD2, was identified in 2002 to be initially expressed in the developing urogenital ridges and later in Sertoli cells and prospermatogonia at around E11.5 (Adams and McLaren 2002) (Figure 1). Expression of the *Ptgds* gene in the developing testis was noted to be in a similar pattern as both *Sry* and *Sox9* starting at the center and moving to the anterior poles in the developing testis (Wilhelm et al. 2007). Furthermore, expression of L-PGDS protein was present in E12.5 male gonads in both Sertoli and germ cells (Moniot *et al*. 2009) that it is regulated upstream through either COX1 (cyclooxygenase 1) or COX2 pathways (Rossitto et al. 2015). However, neither male *Cox2*- nor *Cox1*- knockout mice are infertile; thus, there may be multiple ways to promote production of L-PGDS in order to have a sufficient quantity of PGD2 (Gerena *et al*. 2000a; Gerena *et al*. 2000b). PTGDS is up-regulated by *Sox9* but not *Sry* (Wilhelm et al. 2007). PGD2 acts through its receptor, DP1 (prostaglandin D2 receptor 1) in Sertoli cells, to translocate the cytoplasmic SOX9 protein, to the nucleus to influence gene expression.

How is PGD2 regulated?

Many endocrine disruptors such as phthalates, bisphenol and NSAIDS that inhibit COX activities also reduce PGD2 production as demonstrated in a mouse Sertoli cell line and fetal testis cultures (Kristensen *et al*. 2011a; Kristensen *et al*. 2012; Kristensen *et al*. 2011b). Thus, it appears that the COX signaling pathway is involved and potentially downstream of SOX9 to induce expression of PGD2. In addition to PGD2, COX1 and 2 can stimulate upregulation of other prostaglandins such as PGE2 which is proangiogenic and can further enhance the expression of VEGFA (Cheng *et al*. 1998; Pai *et al*. 2001). Furthermore, there appears to be a positive feedback pathway between COX2 and VEGFA with each of them increasing the other's expression as well as VEGFA upregulating upstream factors in the arachidonic pathway to further enhance COX2 activity. COX1 is thought to be constitutively expressed while most of the effects of hypoxia and VEGFA may be through stimulation of the inducible COX2 (Iniguez *et al*. 2003) (Fig. 2). Since, angiogenic inhibitors do not appear to inhibit COX1; it is possible that the constitutively-expressed COX1 may safeguard some function of angiogenesis within the developing testes.

Mesonephric cell migration directs testis development: are the migrating mesonephric cells peritubular myoid cells or endothelial cells?

As Sertoli cells differentiate and establish intimate contact with germ cells, they form clusters with germ cells aptly named Sertoli-germ cell clusters (Tesarik *et al*. 2002). Germ cells are of extragonadal origin and migrate to the indifferent gonad prior to Sertoli cell differentiation (Mintz and Russell 1957; Ozdzenski 1969). Germ cells do not appear to induce seminiferous cord formation since cord formation still occurs in the testis of c-Kit (kit oncogene, also known as c-Kit) knockout despite a lack of primordial germ cells migration to and colonization of the indifferent gonad (Nocka *et al*. 1989; Runyan *et al*. 2006).

Very elegant studies have demonstrated that mesonephric cells migrating from the mesonephros are necessary for cord formation and later that the developing testis from an indifferent gonad directs mesonephric cell migration (Buehr *et al*. 1993). This migration can even pass through an ovary inserted between the mesonephros and the developing testis (Tilmann and Capel 1999). Initially the migrating cells were thought to be pre-peritubular myoid cells since that cell type is what forms around seminiferous cords and aids later in development of the blood-testis barrier (Buehr et al. 1993). However, others have demonstrated that all cells that migrated are endothelial cells (Combes *et al*. 2009; Cool *et al*. 2008).

Since the Sertoli cells are the first cell to differentiate in the testis (Magre and Jost 1991), they were thought to be the catalyst for induction of endothelial cell migration via secretion of factors that serve as chemoattractants for mesonephric cells. Later it was determined that mesonephric cell migration not only directs seminiferous cord formation but also may induce proliferation of the somatic cells up to a certain threshold to restrict their number. Early studies identified factors that had receptors within PTM cells such as neurotrophins (NT) (Levine *et al*. 2000) and PDGF's (Puglianiello *et al*. 2004). The low affinity receptor for the neurotrophins, NGFR (nerve growth factor receptor), is expressed in cells which appear to migrate from the mesonephros into the differentiating testis and by P0 it is only expressed in a thin layer of cells which appear to have differentiated into the PTM layer that surrounds each seminiferous cord (Russo *et al*. 1999). Other experiments demonstrated that NT3 may be involved in mesonephric cell migration and *Trk* knockout mice contained fewer cords within the testis than wild type mice, in addition to some fused or odd shaped cords (Cupp *et al*. 2002; Cupp *et al*. 2003).

The focus then became the endothelial cells because most of the cells that migrate from the mesonephros contained endothelial cell markers. In addition, sex-specific vasculature develops in the testis and ovary with large differences noted in vascular reorganization and origin in the testis compared to the ovary (Bott *et al*. 2010; Combes et al. 2009; Cool et al. 2008). It was postulated Sertoli cells produced angiogenic or vascular-specific factors to induce endothelial cell migration and cause sex-specific vascular development and seminiferous cord formation (Bott et al. 2010; Tilmann and Capel 1999). Again the question was what angiogenic or vascular-specific factors were produced by the Sertoli cells around the time of sex-specific vasculature development and seminiferous cord formation?

What composes the VEGFA ligand receptor family and how does VEGFA signal through its receptors and co-receptors?

The VEGF family was a logical growth factor family to evaluate its involvement in endothelial cell migration within the developing testis due to its ability to stimulate endothelial cell migration, survival, proliferation and differentiation to initiate vasculogenesis and angiogenesis within developing organs and tumors (Ferrara *et al*. 2003; Lee *et al*. 2015). The VEGF family is composed of VEGFA, VEGFB, VEGFC, VEGFD, and PGF (Placental Growth Factor, previously known as PlGF). VEGFA is the most studied family member and is critical for angiogenic processes (Ferrara et al. 2003). Even the disruption of one allele (heterozygotic deletion) causes embryonic lethality between E11 and

E12. Since neovascularization does not occur in these mice, this suggests that the embryonic lethality is due to a lack of oxygen and nutrients delivery to organs and that VEGFA is critical for most organ functions (Ferrara *et al*. 1996).

What are the receptors to which VEGFA binds?

VEGFA binds to two different tyrosine kinase receptors: kinase insert domain protein receptor (KDR also known as VEGFR2 and FLK1), and FMS-like tyrosine kinase 1 (FLT1, also known as VEGFR1) (Ferrara et al. 2003). Both the KDR and the FLT1 receptors contain an extracellular domain comprised of seven immunoglobulin-like (Ig-like) folds (Fig 3). The function of the Ig-like folds were determined through mutation experiments indicating the VEGFA high affinity ligand binding domain is within the 2nd and 3rd folds while the 1st and 4th Ig-like fold regulate the ability for the ligand to bind and the receptor to dimerize (Davis-Smyth *et al*. 1998; Fuh *et al*. 1998; Shinkai *et al*. 1998; Vieira et al. 2010). The KDR receptor is the primary receptor that VEGFA signals through (Cunningham *et al*. 1999; Kroll and Waltenberger 1997; Seetharam *et al*. 1995; Waltenberger *et al*. 1994; Wheeler-Jones *et al*. 1997). This is mainly due to the observation that the tyrosine phosphorylation sites on the FLT1 receptor (in the regulatory juxtamembrane domain and five within the C-terminus; Figure 3) are never phosphorylated when the VEGFA ligand binds endothelial cells.

FLT-1 receptor—why is it called the decoy receptor?

FLT1 has higher affinity for VEGFA than KDR (Gille *et al*. 2001; Olofsson *et al*. 1998; Waltenberger et al. 1994). Since there was no phosphorylation of its tyrosine residues the main function of this receptor was thought to be to function as a decoy receptor for VEGFA and, thus, to inhibit VEGFA-KDR ligand-receptor interactions and subsequent signal transduction. Presumably this was to allow for inhibition of vascularization of tissues (Ferrara 2000; Zachary and Gliki 2001). This is supported by a study demonstrating that FLT1/VEGFR1 knockout mice that die at E8 and E9 is due to a failure of endothelial cells to assemble into a vasculature circuit (Fong *et al*. 1995). Since most KDR-mediated VEGFA binding results in vascularization of tissues, an increase in FLT1 production to inhibit binding to KDR may be a method to regulate vascularization. Recent reports have also suggested that FLT1 may prevent endothelial cell apoptosis and thus is a potential survival factor (Vieira et al. 2010). This may be due to FLT1 but not KDR being regulated by hypoxia. Within the promoter region of the FLT1 receptor is a binding site for hypoxia inducible factor 1, alpha subunit (HIF1A) (Gerber *et al*. 1997). Thus, FLT1 expression along with upregulation of VEGFA through hypoxia (since there is a HIF1A promoter site in the VEGFA promoter, as well) may allow for survival of these cells and stimulation of some sort of vascular growth to provide oxygen to the tissue. Due to alternative splicing, FLT1 is not only present in a membrane-bound form but also has a soluble form (sFLT1) that acts similarly to binding proteins. Thus this soluble form may also act as a decoy receptor to sequester VEGFA away from KDR (Kearney *et al*. 2004). An increase in sFLT1 has been demonstrated to occur in pathological states such as preeclampsia (Autiero *et al*. 2003; Hiratsuka *et al*. 2001). Thus, regulation of the soluble as well as membrane forms of FLT1 are critical to allow for appropriate vascularization of tissues.

FLT1 signaling pathways

FLT1 contains several potential tyrosine autophosphorylation sites in the c-terminus and also juxtamembrane transmembrane domain. These sites are suppressed by a repressor element located in the juxtamembrane domain which inhibits the phosphorylation of these tyrosine residues after VEGFA binding (Gille *et al*. 2000). However in monocytes and macrophages this repression is not present and there is no information to suggest how this occurs other than secretion of factors that relieve this inhibition. Thus, in these particular cell types, FLT1 can recruit several different accessory proteins such as protein kinases related to SRC (Rous sarcoma oncogene), FYN (fyn proto-oncogene) and YES1 (Yamaguchi sarcoma viral (v-yes) oncogene homolog 1) (Chou *et al*. 2002).

Does FLT-1 signal in the somatic cells of the testis or is it repressed similarly to endothelial cells?

Caires et al. (2012) demonstrated that there was increased phosphorylation of tyrosine (Y) 1333 within the FLT1 receptor in somatic cells at postnatal day 3 (P3) in mice compared to activation in gonocytes, Sertoli, and Leydig cells at P1 (Caires *et al*. 2012). Also inhibitors which blocked both FLT1 and KDR simultaneously were effective in testis organ cultures in inhibiting both sex-specific vascular development as well as seminiferous cord formation (Bott *et al*. 2006) (Table 1). To our knowledge, no experiments have been conducted to determine if FLT1 signals in the somatic or germ cells of the testis early in development, but it is possible that this occurs. Since FLT1 is not present until after seminiferous cord formation in the testis (Bott et al. 2006) it may not be critical. However, since FLT1 can be upregulated by hypoxia, it may be a failsafe mechanism to initiate neovascularization in some tissues outside the testis where VEGFA or KDR are limiting and vascularization of the tissue is critical for survival.

Why is KDR thought to be the primary receptor through which VEGFA signals?

In contrast to FLT1, the KDR receptor is autophosphorylated on several tyrosine residues after ligand binding and receptor dimerization, that have been depicted in the human in the kinase insert domain Figure 3): Y951 (Y949 mouse); in the tyrosine kinase domain: Y1054 and Y1059 (Y1053, Y1057 mouse); and in the C-terminal domain Y1175 and Y1214 (Y1173,Y1212 mouse) (Eliceiri *et al*. 1999; Vieira et al. 2010; Werdich and Penn 2005). Most phosphorylation experiments have been conducted in endothelial cells; however, these tyrosine residues recruit different downstream signaling molecules when phosphorylated to induce different events (Eliceiri et al. 1999; Vieira et al. 2010; Werdich and Penn 2005). This autophosphorylation is actually a form of trans-phosphorylation that is prevented until the conformation of KDR changes following VEGFA binding and the receptor monomers come into close juxtaposition (Tao *et al*. 2001).

KDR phosphotyrosines such as Y951 and Y1175 are recognized by SRC family kinases which have been shown to modulate endothelial proliferation and migration (Eliceiri et al. 1999; Vieira et al. 2010; Werdich and Penn 2005). Many different knockout mouse models have been developed which mutate or eliminate these tyrosine residues in order to determine their function (Figure 3). The Y951 phosphotyrosine works through TSAd (T Cell–Specific Adapter) and Src and is involved in endothelial cell permeability and migration while both

Y1175 and Y1214 also are involved in endothelial cell proliferation or migration as indicated through mutation experiments (Rajagopal *et al*. 1999). The tyrosine residue Y1214 works through growth factor receptor bound protein 2 (GRB2) and has been implicated to be in control of actin reorganization and cell migration which is thought to occur through CDC42 (cell division cycle 42) and MAPK (mitogen-activated protein kinase) cascades (Lamalice *et al*. 2004) (Figure 3). Many different signal transduction pathways occur through Y1175: activation of phospholipase C (PLC) gamma, protein kinase C (PKC), and MAPK to cause proliferation. Src is recruited to this residue as well as SHB (src homology 2 domain-containing transforming protein B) which will allow for migration, survival and permeability of endothelial cells (Aouadi *et al*. 2006). SHB controls endothelial cell migration through focal adhesion kinase (FAK) in a PI3 (phosphoinositide-3) kinase-mediated pathway (Kriz *et al*. 2006) (Figure 3). Caires et al., 2012 demonstrated that during early postnatal periods, the 1054 phosphotyrosine is autophosphorylated (1053 in the mouse), and VEGFA signaling through KDR is clearly present in both Sertoli, Leydig and germ cells at different stages of early postnatal development (Caires et al. 2012). Thus, these pathways should be explored to determine which phosphotyrosines are specifically necessary for VEGFA's function within the developing testis during sex-specific vascularization.

KDR is critical to embryogenesis since KDR knockout mice die at E8.5 to E9.5 (Shalaby *et al*. 1995). These embryos lack blood island formation in the yolk sac and experience a dramatic reduction in haematopoetic precursors, suggesting KDR is necessary for vasculogenesis. Furthermore, since they mark the early haemangioblast that differentiates into endothelial cells as well as haematopoeitic progenitors, it is possible that KDR is also involved in haematopoiesis as well as vascularization of tissues through endothelial cells (Ciau-Uitz *et al*. 2013; Jaffredo *et al*. 2005; Medvinsky *et al*. 2011; Murray 1932). Interestingly, mice carrying only a single amino acid mutation that prevents KDR activation of ERK 1/2 die *in utero* due to vascular defects suggesting that phosphorylation of this receptor is critical to multiple vasculature functions within the developing embryo (Sakurai *et al*. 2005).

What is the expression pattern of the KDR, FLT-1 receptors during testis development?

Expression of KDR occurs relatively early and marks sex-specific vascular development in both the testis and ovary as indicated in KDR-LacZ transgenic mice (Bott et al. 2010). Expression of KDR is apparent on cells which appear to be migrating into the testis at the time of sex-specific migration. Later as seminiferous cords form and are apparent, these KDR-LacZ marked cells are present around the seminiferous cords (Bott et al. 2010; Bott et al. 2006). Since the KDR receptor signals through tyrosine kinase autophosphorylation, several different tyrosine kinase inhibitors were utilized to determine how this may affect seminiferous cord formation and sex-specific vascular development in testis organ cultures. In rat testis organ cultures, VEGFRTKI (a tyrosine kinase inhibitor specific to KDR and FLT1) inhibited sex-specific vascular development (reduction by 90% in all vascular development in the testis; Table 1) and seminiferous cord formation (Bott et al. 2006). In

addition, a KDR specific inhibitor, SU1498, reduced vascular development by 90% and perturbed cord formation (Bott et al. 2006) (Table 1). Furthermore, KDR-LacZ-positive staining in mouse testis organ cultures was reduced at day 1, 2 and 3 of culture by 66%, 90% and 99%, respectively, when they were treated with VEGFR-TKI. These data suggest that endothelial cell migration, proliferation and survival may have been blocked which prevented the testis from being vascularized (Bott et al. 2010). Thus, it does appear that KDR-LacZ marked cells migrate into the testis and can be inhibited by a specific VEGFR inhibitor. Other inhibitors to PI3 kinase have also been demonstrated to detriment sex-specific vascular development but only reduce vascular development by 56% (Bott et al. 2006) despite perturbing seminiferous cord formation (Bott et al. 2006; Uzumcu *et al*. 2002b) (Table 1). Inhibition of the MAPK pathway also alters cord formation (Uzumcu et al. 2002b), but vascular development was not evaluated. Since VEGFA signals through KDR to stimulate both MAPK and PI3 kinase, these data support of a role for VEGFA in regulating sex-specific vascular development within the developing rodent testis.

FLT1, however, is not expressed in the rodent until after cord formation in the male (Bott et al. 2006) while both KDR and FLT1 are expressed early in the ovary (McFee *et al*. 2009). KDR, FLT1 and VEGFA are expressed by oocyte cysts, oocytes and pregranulosa/granulosa cells of all stage follicles at P0, P3, and P10 as well as in theca cells of late antral follicles (McFee et al. 2009). There is a possibility that FLT1 expression in the ovary sequesters the VEGFA ligand and prevents it from binding to KDR and to block endothelial cell migration within the ovary at the time sex-specific vascular development occurs in the testis.

What are the alternatively spliced products of VEGFA and what are their function(s)?

Of the 25,000 genes in the human genome, at least 70% are alternatively spliced, and abnormal alternative splicing can be correlated with a large number of human genetic deficiencies (Black 2003; Matlin *et al*. 2005). VEGFA is transcribed from a single gene that is alternatively spliced into many different isoforms depending on the cell type of interest. Many VEGFA isoforms have been identified (VEGFA111, 121, 165, 148, 145, 183, 189, 206), and the most common VEGFA isoforms that have been studied are human angiogenic VEGFA206, VEGFA189, VEGFA165, VEGFA145 and VEGFA121 (Figure 4). The numbers on the isoforms denote the number of amino acids and in the rodent there is one less amino acid for each isoform: VEGFA205, VEGFA188, VEGFA164, VEGFA144, VEGFA120 (Fig. 4). For this review we will utilize the rodent VEGFA isoforms since much of the research discussed will be in the rodent (Giacca 2010; Hoeben *et al*. 2004; Kajdaniuk *et al*. 2011).

Within the *Vegfa* gene, there are 8 exons and all VEGFA isoforms contain exons 1–5 except for a newly identified isoform, VEGFA111, which contains only 1–4 and exon 8. A signal peptide is located within exons 1 and 2 and upon cleavage, produces a mature peptide. In exon 5, there are cleavage sites for plasmin and matrix metalloproteinases. Since VEGFA111 does not contain exon 5, this isoform is resistant to their cleavage and is a critical compound utilized in therapies for different cell ischemia (Houck *et al*. 1991; Mineur *et al*. 2007). Exons 6a and 7 encode heparin binding domains and isoforms

containing these exons bind to cell surfaces and are not as diffusible as isoforms that do not contain these exons. Also contained within exons 6a and 7 are a neuropilin binding site (Keyt *et al*. 1996; Lee *et al*. 2005). The 5' untranslated region (UTR) of *Vegfa* is approximately 1038 nucleotides in humans and consists of 3 CUG start codons, an AUG initiation codon and two internal ribosome entry sites (IRESes) which can control VEGFA translation. The 3'UTR consists of polyadenylation signals and AU-rich response element which allows for response to stabilizing and destabilizing mRNAs (Konopatskaya *et al*. 2006; Seifi *et al*. 2012) (Figure 4).

The VEGFA205 isoform contains all exons and is proposed to bind more tightly to extracellular matrix proteins in addition to being greater in amino acid number than other isoforms. The VEGFA188 isoform has 2 heparin binding domains, remains relatively close to the cell in which it was secreted, and acts locally to support branching of vasculature. The VEGFA164 isoform contains only one heparin binding domain and has intermediate diffusibility, and has a prominent role in the formation of large blood vessels. The VEGFA120 isoforms lacks a heparin binding domain, is the most diffusible, and can migrate the farthest from the secreted cells. The differences in diffusibility of these different VEGFA isoforms allow for the development of a chemoattractant gradient which induces endothelial cells to migrate toward cells which produce VEGFA (Carmeliet and Collen 1999; Ferrara 2010; Grunstein *et al*. 2000; Krilleke *et al*. 2009; Simons 2007; Veikkola and Alitalo 1999).

What is the expression and potential actions of VEGFA isoforms in the developing testis?

The presence of multiple VEGFA isoforms differing in number of amino acids was identified in the developing rat testis: VEGFA120, 164 and 188 (Bott et al. 2006) (Figure 4). Using a pan antibody to all VEGFA isoforms it was demonstrated that protein for the pan-VEGFA (which detects both angiogenic and antiangiogenic isoforms) was lower at E13 and increased by P4. Sertoli cells had the greatest abundance of panVEGFA expression around the time of cord formation (Bott et al. 2006).

It does not appear that a chemoattractant gradient for VEGFA exists in the ovary around the time of seminiferous cord formation in the rat testis because the only VEGFA isoforms that are present are VEGFA164 and VEGFA120. The two higher molecular weight VEGFA isoforms VEGFA188 and VEGFA205 have not been detected (McFee et al. 2009); thus, we hypothesize that there is no viable chemoattractant gradient to allow for endothelial cell migration from the mesonephros into the ovary.

Furthermore, treatment of E13 rat testis organ cultures with VEGFA120 and VEGFA164 causes formation of altered (absent, swollen and misshaped) seminiferous cords as well as dramatically increased vascular density (160 and 157% that of controls; Table 2). Thus, excess VEGFA angiogenic isoforms 164 and 120 can elicit detrimental effects on testis development and potentially have as dramatic effects as inhibition of VEGFA signal transduction discussed earlier (Bott et al. 2006) (Table 1). All of these VEGFA angiogenic isoforms have been shown to bind both KDR and NRP1, but even though there may be binding of VEGFA120 (which does not have a heparin and thus NRP1 binding domain) to

NRP-1, the bridging to KDR does not appear to occur. This may affect the amplification of the VEGFA120 signal transduction as well as the recycling of the KDR receptor within the cell (Pan *et al*. 2007).

VEGFA antiangiogenic isoforms

In 2002, a second set of sister isoforms was identified through alternative splicing and replacement of exon 8a with 8b (Bates *et al*. 2002) (Figure 4). These sister VEGFA isoforms were considered inhibitory to angiogenic functions and were labeled antiangiogenic VEGFA isoforms. Several antiangiogenic isoforms have been identified- VEGFA212b, VEGFA145b, 183b, 189b, 165b, and 121b (Bates et al. 2002; Dehghanian et al. 2014; Woolard *et al*. 2004) (Fig. 3). VEGF165b is normally expressed in tissues but is down-regulated in prostate and renal tumors suggesting a role in inhibiting the vasculogenic and angiogenic actions of other VEGF isoforms (Bates et al. 2002; Woolard et al. 2004). Several reports have suggested that these antiangiogenic VEGFA isoforms may function to antagonize angiogenic VEGFA isoform functions through binding of the receptors and preventing angiogenic isoforms from binding (Qiu et al. 2009).

Much controversy has ensued over whether these “b” isoforms exist or whether they are artifacts of alternative splicing (Harris *et al*. 2012). While it is true that determining the difference in the angiogenic vs the antiangiogenic isoforms has been a challenge, data from our laboratory and others using *either* recombinant angiogenic *or* antiangiogenic VEGFA isoforms has resulted in very different outcomes that demonstrate the existence of divergent VEGFA isoforms (Artac *et al*. 2009; Baltes-Breitwisch *et al*. 2010; Bates *et al*. 2013; Caires et al. 2012; McFee et al. 2009).

For instance, the VEGFAxxxb antibody (detects all antiangiogenic isoforms but sold as antiVEGFA165b) detected antiangiogenic isoforms in germ cells from E14 to E16 with expression in the interstitium by P0 (Baltes-Breitwisch et al. 2010). This result in expression was very different from the panVEGFA antibody which detects all VEGFA isoforms where expression was detected in Sertoli cells and potentially some germ and Leydig cells (Bott et al. 2006). When we used this antibody to the exon 8b (which will neutralize antiangiogenic isoforms and allow endogenous VEGFA angiogenic isoforms to bind) on E13 rat testis organ cultures we had similar results as treating with recombinant VEGFA120 or VEGFA164 (Table 2). Either dose of VEGFAxxxb antibody, dramatically increased vascular density (195%) versus control testes compared to increases in vascular density of 160% (VEGFA164) or 157% (VEGFA120) (Table 2). In addition, the seminiferous cords were swollen, perturbed, or misshapen which was very similar to what was seen with the recombinant angiogenic VEGFA isoforms treatment (Baltes-Breitwisch et al. 2010). Thus, it appears that removal of the antiangiogenic isoforms increases the amount of endogenous angiogenic VEGFA isoforms that can bind to the VEGFA receptors and elicit similar effects to treatments with recombinant angiogenic VEGFA isoforms. Thus, from our data and others we would suggest that the antiangiogenic isoforms do exist whether they are produced from alternative splicing or through other recently identified means.

Whole testis western blots conducted with the antiangiogenic VEGFAxxxb antibody did not demonstrate major differences in the levels of antiangiogenic VEGFA isoform abundance

during early testis development, but the immunohistochemistry clearly indicated that VEGFAxxxb isoforms were not expressed in somatic cells and only in germ cells during early testis development (Baltes-Breitwisch et al. 2010). Quantitiative PCR demonstrated a 5-fold greater abundance of antiangiogenic VEGFA165b in the ovary vs the testis at E13 which indicated that antiangiogenic isoforms may be inhibiting angiogenic VEGFA isoform functions related to endothelial cell migration or development of chemoattractant gradients.

Recently, a paper was published in Cell (Eswarappa *et al*. 2014) which identified yet another antiangiogenic VEGFA isoform in human and bovine endothelial cell lines that is produced by programmed translational readthrough which has a cis-acting element in the *Vegfa* 3'UTR that serves a dual function (Figure 4). It can encode the shortened peptide but can also direct the programmed readthrough by decoding the UGA stop codon (Figure 4). A heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 binds this element and promotes readthrough, producing a product that has exon 8a and 8b. This product acts similarly to antiangiogenic isoforms and is down regulated in certain cancers (Eswarappa et al. 2014). The authors of this paper have named this isoform VEGFAx. The Ax is named for the Ax element which is a 62 nucleotide extension inserted between the 2 stop codons which normally would confer either angiogenic (with exon 8a) or antiangiogenic isoforms (with exon 8b; Figure 4). Thus, the binding protein hnRNP A2/B1 binds the A2/B1 response element (A2RE) in the Ax element and positively regulates VEGFA readthrough. This finding is exciting since this is the first time that a trans factor has been identified that regulates translational readthrough. Furthermore, most translational readthrough was only thought to occur in evolutionarily lower-ordered species such as Drosophila, viruses, etc. Since the VEGFAx protein acts like an antiangiogenic isoform, more experiments are needed to determine if this protein is present only in certain cell types that produce this trans-factor hnRNP A2/B1 or whether they are ubiquitous (Eswarappa et al. 2014).

In experiments where VEGFA isoforms have been subcloned from primary bovine granulosa cells, some VEGFA isoform PCR subcloned products contained both exon 8a and 8b suggesting that there is a readthrough product in the bovine (McFee, Spuri Gomes, Artac, Summers, Pohlmeier, Brauer, Kurz, Cushman, Wood and Cupp, unpublished observations) (Figure 4). This was not the case for subcloned VEGFA from rat granulosa or Sertoli cells. In addition, there were subcloned products which only contained either the 8a or 8b in the bovine and rat without the Ax product. Thus, within each cell, the necessary machinery-whether it is splicing proteins or proteins that allow for programmed read through- may be present at critical physiological states to ensure the appropriate balance of VEGFA angiogenic and antiangiogenic isoforms. More experiments are needed to ensure the VEGFAx product exists; however, if these data are confirmed there could be an evolutionary change in insertion of the Ax sequence (62 nucleotides) which may provide different functions in higher evolved mammals.

Are there differences in VEGFA angiogenic and antiangiogenic isoform actions on their receptors?

Neuropilin 1 (NRP1) was originally identified as a receptor in the nervous system and was found to modulate semaphorin signals (He and Tessier-Lavigne 1997; Takahashi *et al*. 1998)

and direct neuronal axon guidance. However, NRP1 has also been found to be critical for vascular development in cooperation with the VEGFA angiogenic isoforms in many different angiogenic processes (Kawasaki *et al*. 1999). NRP1 knockout mice die between 10.5 and 12.5 and have vasculature development defects as well as heart and neural defects suggesting that NRP1 is critical for both vascular and neuronal development (Kawasaki et al. 1999). NRP1 can bind to semaphorins instead of VEGFA and this impairs endothelial cell migration (Miao *et al*. 1999). Semaphorins are present within the testis and appear to be secreted by Sertoli cells (Perala *et al*. 2005). Whether semaphorins are antagonistic to vascular development within the testis this is yet unknown. Alternatively, NRP/VEGFA complexes binding to FLT1 can also prevent endothelial cell migration and vascular development (Miao et al. 1999) so there are potentially many check points that are present within the testis to prevent over-vascularization to ensure development of the seminiferous cords.

NRP1 is a co-receptor to VEGFA but it does not bind either KDR or FLT-1 but instead binds to a region on the VEGFA ligand (Figure 4). Because KDR lacks a PDZ-binding domain it cannot interact with a PDZ protein synectin (which is also called GIPC1 or NIP). NRP1 has a SEA motif which allows for PDZ protein synectin binding. KDR can utilize NRP1 bound to VEGFA angiogenic isoforms as a bridge to enhance signal transduction and also allow for appropriate trafficking of NRP1 within the cell to allow for receptor recycling (Cai and Reed 1999; Wang *et al*. 2006).

Due to the presence of exon 8b rather than 8a, the VEGFA antiangiogenic isoforms cannot bind NRP1 since much of their binding region is within the exon 8a region. Thus, elimination of NRP1 or overexpression will only affect angiogenic VEGFA isoform functions (Kawamura *et al*. 2008). When NRP1 is bound to VEGFA164 and then they bind and signal through KDR, this causes the KDR receptor to be recycled through Rab5, Rab4 and Rab11 vesicles. However, antiangiogenic VEGFA cannot bind NRP1. Therefore, when VEGFA164b binds to KDR, KDR bypasses the Rab11 vesicles in cells and is routed to the Rab7 vessicles which moves KDR to the degradation pathway. Also, if the SEA binding motif for synectin is deleted from NRP1, will attenuate the receptor-ligand signal amplification since the SEA carboxyterminal domain of NRP1 mediates the events to allow for KDR recycling via Rab11 positive endosomes (Ballmer-Hofer *et al*. 2011).

How are NRP1 receptors expressed and regulated within the gonad?

NRP1 receptors are present early in both the testis and ovary (Walker, Lu, Clopton, and Cupp, unpublished observations) and have the potential to interact with VEGFA and present both to KDR and FLT1. Because FLT1 is present during gonadal development, it is possible that FLT1 is sequestering NRP1 and VEGFA away from KDR and limiting signal transduction and subsequent endothelial cell migration in the ovary (Baltes-Breitwisch et al. 2010). An inhibitor to the NRP1 co-receptor, Je-11, was utilized to determine its effects on E13 rat testis organ cultures. Initially this inhibitor was thought to be specific only to KDR but later was found to inhibit only NRP1. This inhibitor caused a 42% reduction in vascular development with a less defined coelomic vessel and cords that were either not present or perturbed (Bott et al. 2006) (Table 1). This inhibitor was not as effective as the inhibitors

either to KDR alone or to KDR and FLT1 simultaneously, suggesting that KDR can signal and cause vascular development independent of NRP1 which has been demonstrated in many different endothelial cells and systems with vascularization (Vieira et al. 2010). However these results do demonstrate its role in enhancement of KDR signaling. Through altered receptor recycling we can envision a function for NRP1 in development of endothelial cell migration, arteriogenesis–mainly the coelomic vessel-, and induction of cord formation within the developing testis.

What happens *in vivo* when VEGFA isoforms are eliminated in somatic and germ cells during testis development?

In order to determine if VEGFA was critical for testis development multiple conditional knockout mouse lines were developed to eliminate all VEGFA isoforms in: Sertoli and some germ cells (*pDmrt1-Cre;Vegfa*$^{-/-}$); Sertoli and Leydig cells (*Amhr2-Cre;Vegfa*$^{-/-}$); and Sertoli cells only (*Amh-Cre;Vegfa*$^{-/-}$ and *Sry-Cre;Vegfa*$^{-/-}$). To date, testes formed normally in these male mice by P0 and there do not appear to be differences in vasculature based on published work from our laboratory (Lu *et al*. 2013) as well as on unpublished results (Sargent, Spuri Gomes, Essink, Kurz, Pohlmeier, Ferrara, Bouma, McLean, and Cupp; Sargent, Essink, Bremer, Pohlmeier, and Cupp, unpublished observations) in VEGFA or NRP1 knockout versus the control testes. Instead of effects on vasculature, our laboratory has demonstrated effects on male fertility through altered spermatogonial stem cell maintenance (Lu et al. 2013). Furthermore, using different VEGFA angiogenic and antiangiogenic isoforms we also have demonstrated different effects on number of spermatogonial stem cells in endogenously depleted recipients (Caires et al. 2012). It is possible that vascular development in these males is delayed embryonically; however, what we can state is that VEGFA does not appear to be required for testis development since these testes contain a coelomic vessel and seminiferous cords that form into seminiferous tubules. Furthermore, from the P0 time period the only phenotype that appears to be abnormal is the number of germ cells at different stages and the number of undifferentiated spermatogonia leading to differences in number of sperm (Lu et al. 2013). These findings were echoed by measurement of VEGFA in roe deer where expression correlated more with transition of germ cell stages than with testis microvasculature (Wagener *et al*. 2010). When *Vegfa* was in control of and overexpressed by the MMTV promoter in mice, the animals were infertile. This infertility was thought to result, in part, from testicular spermatogenic cell arrest which further demonstrates non-endothelial effects of VEGFA in the testis (Korpelainen *et al*. 1998).

Due to our conditional knockout mouse data it does not appear that VEGFA is absolutely required for normal testis development. In many ways this makes sense because throughout testis development there are multiple factors that upregulate expression of genes to ensure proper differentiation of a testis. NR5A1 induces initial expression of *Sox9* which is then upregulated by SRY and SOX9 (Sekido and Lovell-Badge 2008) and SOX9 not only upregulates FGF9 and PTGS but it also upregulates itself (DiTacchio et al. 2012). A role of both PTGS and FGF9 is to continually provide sustained expression of SOX9 (Rossitto et al. 2015) (Fig. 1). Thus, since sex-specific vascular development drives seminiferous cord

formation it would logically follow that there are multiple mechanism which allow for this to happen– potentially in the absence of one such as VEGFA.

What are some potential alternative pathways to initiate endothelial cell migration and sex-specific vascularization that do not utilize VEGFA?

Cox1/2 pathway

VEGFA is regulated through increased HIF1A (hypoxia inducible factor 1, alpha subunit) upregulation during hypoxia. It is possible that the COX pathway through PGE2 upregulates VEGFA as well as direct upregulation through COX2 (Fig. 2). Postnatal day 30 whole testes were collected from *Sry-Cre;Vegfa*$^{-/-}$ conditional knockout mice and compared to controls for differences in genes within the VEGFA signal transduction pathway using a VEGFA signal transduction array plate. When the data analyzed with Ingenuity Pathway Analysis (IPA), the COX1 (PTGS1) constitutively expressed gene was upregulated 5-fold (Sargent, Spuri Gomes, Essink, Kurz, Pohlmeier, Ferrara, Bouma, McLean, and Cupp; Sargent, Essink, Bremer, Pohlmeier, and Cupp, unpublished observations). These data indicate that elimination of VEGFA (in this case in Sertoli cells) may allow for compensatory mechanisms which regulate other angiogenic factors to allow for normal tissue development.

Other VEGF ligands

VEGFB knockout mice are fertile and viable with no apparent phenotype; however, they do exhibit a subtle cardiac phenotype (Aase *et al*. 1999). Transcription of the *Vegfb* gene is not stimulated by hypoxia or other growth factors, cytokines, etc. VEGFB binds to FLT1 and NRP1, while VEGFC binds to VEGFR3 and KDR and enhances vasculature within the lymphatic system. VEGFC is expressed in the heart, placenta, ovary, small intestine and thyroid gland (Olofsson *et al*. 1999). In the embryo, it is expressed in regions where lymphatic vessels sprout from embryonic veins. Similar to VEGFB this gene is not regulated by hypoxia but interleukin 1 (IL1) and tumor necrosis factor (TNF) can increase VEGFC (Kukk *et al*. 1996). VEGFD is expressed in the lung, heart, skeletal muscle, colon and small intestine. In embryos, VEGFD is most abundant in the lung. Like VEGFC, it binds to both VEGFR3 and KDR (Olofsson et al. 1999). There have been no reports of VEGFB or VEGFC present in or involved with testis development, but due to their potential actions through VEGF receptors, these could be two potential candidates that may function in VEGFA's absence to initiate sex-specific vascular development.

What about other growth factors that have similar functions to VEGFA that could compensate for sex-specific vascular development?

Platelet Derived Growth Factor

Platelet derived growth factor receptor alpha (PDGFRα) and PDGFRβ are receptors for PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD and these receptors are structurally similar to the VEGFR's (Heldin 2013). PDGFB is expressed in endothelial cells and *Pdgfb* and *Pdgfrb* knockout mice are embryonic lethal during late gestation due to widespread

hemorrhaging and capillary dilation (Hellstrom *et al*. 1999; Leveen *et al*. 1994; Lindahl *et al*. 1997; Soriano 1994). Fetuses appear normal, but the absence of vascular smooth muscle cells and /or pericytes leads to cardiovascular complications. In the rat, PDGF-AA, PDGF-BB and their receptors are expressed in human fetuses (Basciani *et al*. 2002), and PDGF-BB, when inhibited, has been demonstrated to affect vascular development and perturb or totally inhibit seminiferous cord formation in the mouse (Puglianiello et al. 2004). Inhibitors to the PDGF-BB signal transduction pathway (PGFR-specific inhibitor Tyrophostin) have also inhibited seminiferous cord formation in the rat (Uzumcu *et al*. 2002a). Furthermore, PDGF has also been identified to work cooperatively with VEGFA in vascular events and remodeling within the testis (Cool *et al*. 2011) during the process of sex-specific vascular development. Thus, PDGF and its receptors may comprise a mechanism where endothelial migration and seminiferous cord formation occur in the absence of VEGFA isoforms.

R-spondin homolog

Recently, RSPO1 (R-spondin homolog), which is necessary for ovarian development, has been shown to induce angiogenesis within the developing testis and to inhibit actions of the dickkopf homolog 1 (*Dkk1*) gene which perturbs endothelial branching by inhibiting PDGFB (Caruso *et al*. 2015). Interestingly, RSPO1 can rescue DKK1 inhibition of angiogenesis indicating that perhaps RSPO1 is critical for necessary availability of PDGFB for endothelial cell branching during testis development. These actions of RSPO1 are thought to be mediated through beta catenin (CTNNB1) since testes treated *in vitro* with RSPO1 exhibit enhanced nuclear translocation of CTNNB1 which will affect gene expression within the nucleus (Caruso et al. 2015).

VEGF Receptors that bind other ligands

It is possible that the receptors that VEGFA signals through may elicit effects following binding by other ligands within the VEGF family to induce endothelial cell migration. An example of this is the potential for FLT1 to be induced through hypoxia and cooperate with PGF (Placental Growth Factor to promote pathological angiogenesis that occurs during diseases and disorder such as pre-eclampsia (Autiero et al. 2003; Hiratsuka et al. 2001). FLT1 upregulation can be stimulated by hypoxia (Gerber et al. 1997) and has been shown to activate PGF in VEGF-responsive monocytes which release proangiogenic factors. Further, FLT1 tyrosine kinase signaling mediates chemotactive macrophage migration in response to PGF and both VEGF, and PGF promotes macrophage survival during tumor angiogenesis (Adini *et al*. 2002; Barleon *et al*. 1996; Clauss *et al*. 1996; Hiratsuka *et al*. 1998; Selvaraj *et al*. 2003). Recently macrophage migration was demonstrated to occur during testis development and the authors implicated that VEGFA was expressed by macrophages and that vascular development may, thus, be directed by these macrophages (DeFalco *et al*. 2014). These are intriguing data and may suggest that macrophages derived from the yolk sac (which are extraembryonic along with primordial germ cells) are critical for sex-specific vascular development. So the possibility of macrophages directing endothelial cell migration is a potential mechanism that should be further evaluated.

Summary

While there is overwhelming *in vitro* evidence to support the theory that VEGFA and its receptors, FLT1, KDR, and NRP1, are critical for sex-specific vascular development which can allow for seminiferous cord formation, conditional knockout data in mice where VEGFA was eliminated in Sertoli, Leydig and germ cells do not support this theory. Because the development of the testis is so critical to the propagation of the species, suggesting that VEGFA can only be the critical regulator of this process may be somewhat simplistic. Just as SOX9 has multiple regulators which sustain its expression, there are potentially many independent angiogenic pathways which contribute to the migration of endothelial cells to initiate seminiferous cord formation. In this review article, we have suggested several other pathways which may independently mimic the actions of VEGFA in supporting endothelial cell migration and development of this quite extraordinary vascular development within the testis. We, along with others, investigating this area will have many more years to work on unraveling the complexities of this pathway, to gain a better understanding of how the testis develops, and paving new avenues for treating infertility which may be a predictor of male health and lifespan.

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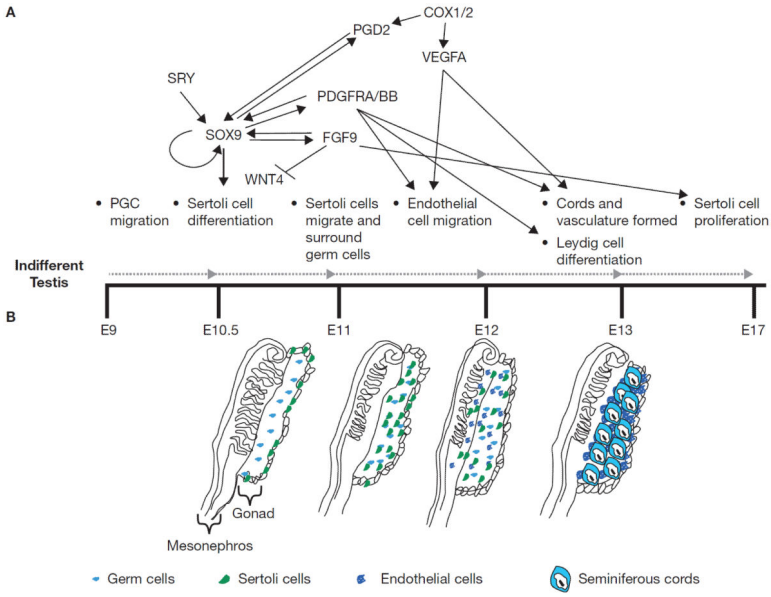

Figure 1.

Gene expression during testis morphogenesis. Depicted above the timeline is a network pieced together based on gene and protein interactions in the literature related to sex-specific vascular development and sexual differentiation. The bottom of the figure depicts different stages of testis development. PGC = primordial germ cell, SRY = sex determining region of Chr Y, SXO9 = SRY (sex determining region Y)-box 9, WNT4 = wingless-type MMTV integration site family, member 4, PGD2 = prostaglandin D2, PDGFR = platelet derived growth factor receptor, FGF9 = fibroblast growth factor 9, COX = cyclooxygenase 1/2, VEGFA = vascular endothelial growth factor A.

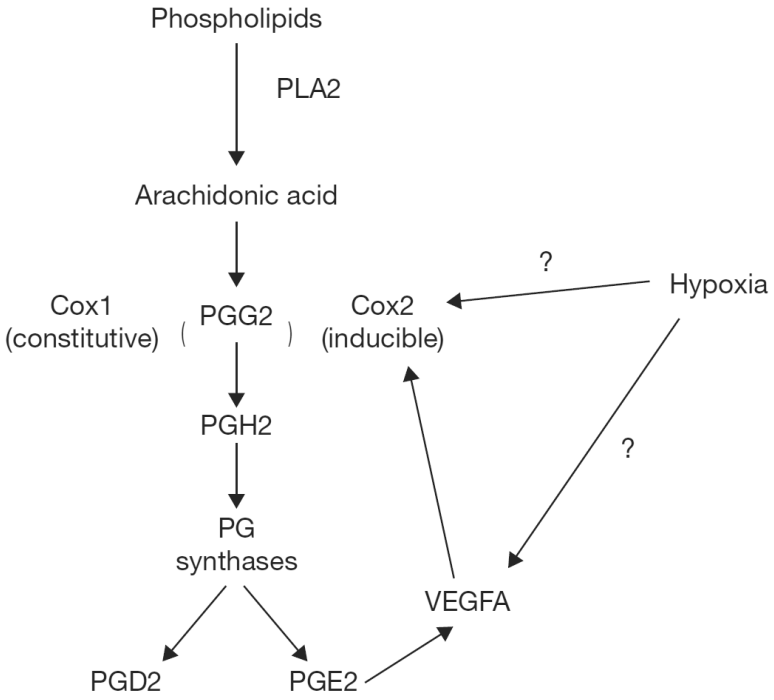

Figure 2.

Proposed pathway of prostaglandin synthesis and interaction with the cyclooxygenase pathways and VEGFA. PLA2 = phospholipase A2, PG = prostaglandin, COX= cyclooxygenase, VEGFA = vascular endothelial growth factor A.

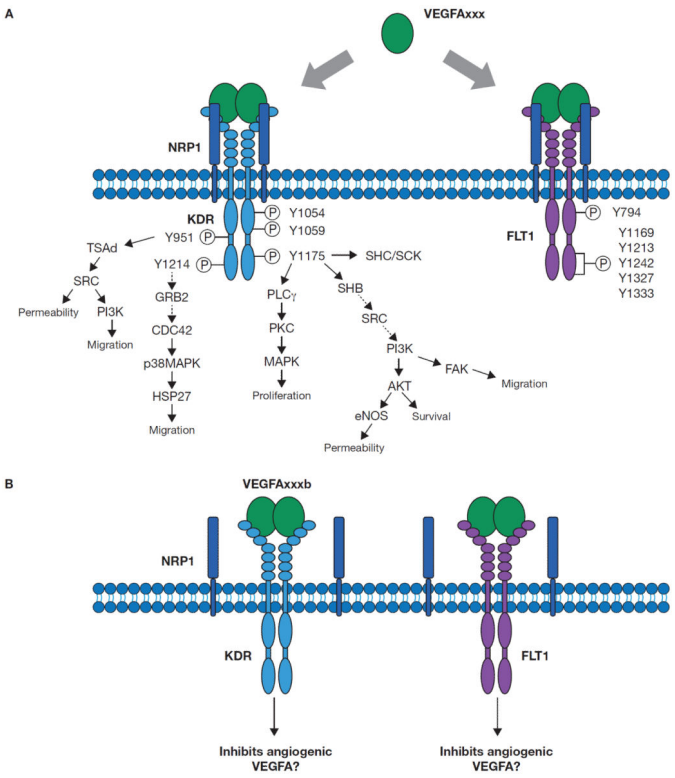

Figure 3.

VEGF receptors which are critical to the actions of VEGFA. Pictured is binding of angiogenic VEGFA isoforms to either KDR (top right) or FLT1 (top left). Only angiogenic isoforms of VEGFA bind to NRP1 (top). Antiangiogenic VEGFA isoform binding to either KDR (bottom left) or FLT1 (bottom right) results in less and different signal transduction than angiogenic. Antiangiogenic VEGFA isoforms are unable to bind NRP1 (bottom). Phosphorylation sties are depicted as “P” within a circle. Y = tyrosine, VEGFA = vascular endothelial growth factor A, KDR = kinase insert domain protein receptor, FLT1 = fms-like tyrosine kinase 1, NRP1 = neuropilin 1, TSAd = T-cell Specific Adaptor, SRC = Rous sarcoma oncogene, PI3K = phosphatidylinositol 3-kinase, GRB2 = growth factor receptor bound protein 2, CDC42 = cell division cycle 42, (p38)MAPK = mitogen-activated protein kinase, HSP27 = heat shock protein 27, PLCγ = phospholipase C gamma, PKC = protein kinase C, SHC/SCK = src homology 2 domain-containing transforming protein, SHB = src homology 2 domain-containing transforming protein B, AKT = thymoma viral proto-oncogene, eNOS = nitric oxide synthase 3, endothelial cell, FAK = focal adhesion kinase.

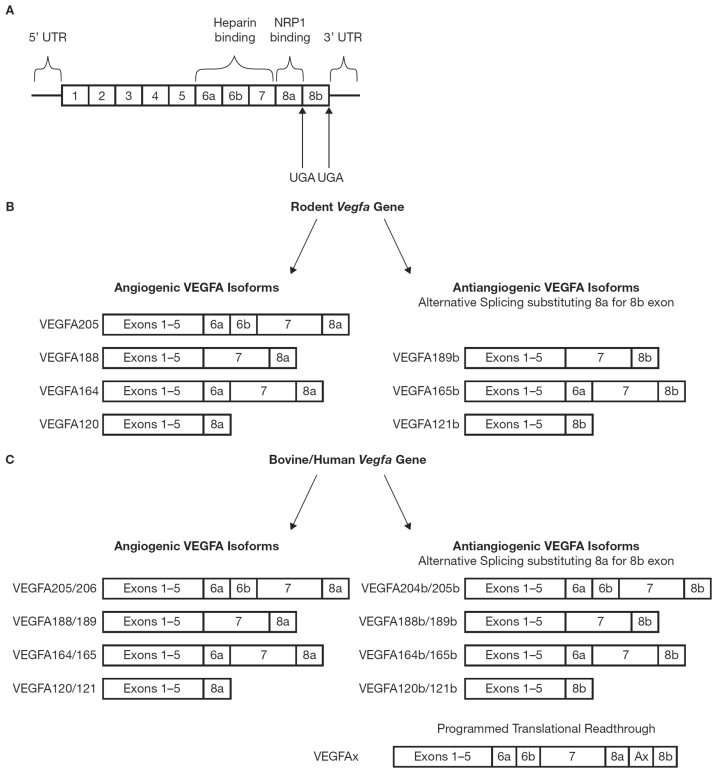

Figure 4.

VEGFA gene containing all exons and both heparin- and NRP1-binding sites as well as the canonical stop codons targeted for alternative splicing between either angiogenic or antiangiogenic isoforms (A). Various rodent angiogenic VEGFA isoforms (B, left) and antiangiogenic isoforms (B, right) have been identified following alternative splicing. In the human and bovine, the amino acids numbers differ from rodent. Pictured are the most well known/studied angiogenic VEGFA isoforms (left, C) and antiangiogenic isoforms (top right, C). Also depicted is the recently identified VEGFAx that possesses antiangiogenic properties in humans and bovines (bottom right, C).

Table 1

Signal Transduction Inhibitors of VEGFA Signaling and their effects on Rat Embryonic Testis Cord Formation and Vascular Development *in vitro* (*Data from* Bott *et al*. 2010)

Signal Transduction Pathway	Inhibitor	Effect on Cord Formation	Effects on Vasculature	PECAM1 Staining
Inhibits KDR	SU1498 (Calbiochem, #572888)	No cord formation	Little vasculature, no coelomic vessel	10%
Inhibits KDR and FLT1	VEGF-TKI (Calbiochem, #676475)	No cord formation	No vasculature dividing, no coelomic vessel	10%
Inhibits NRP1	Je11 (Calbiochem, #676494)	No cords/ perturbed cord formation	Coelomic vessel, some microvessels, but reduced density	58%
Inhibits PI3K	LY294002 (Calbiochem, #440202)	Cords perturbed or swollen	Weak coelomic vessel, less dense vasculature	54%

VEGFA = vascular endothelial growth factor A

KDR = kinase insert domain protein receptor

FLT1 = FMS-like tyrosine kinase 1

NRP1 = neuropilin 1

PI3K = phosphatidylinositol 3-kinase

PECAM1 = platelet/endothelial cell adhesion molecule 1

Table 2

Effects of Recombinant VEGFA Protein or VEGFAxxxb Antibody on Rat Embrynic Testis Testis Cord Formation and Vascular Development *in vitro* (*Data from* Baltes-Breitwisch *et al*. 2010)

Recombinant VEGFA Protein	Effect on Cord Formation	Effects on Vasculature	PECAM Staining
VEGFA120	Swollen cords with some perturbed (R&D Systems, #494-VE)	Thickened coelomic vessel, more vasculature between cords	157%
VEGFA164	Some cords misshapen or perturbed (R&D Systems, #564-RV)	Thickened coelomic vessel, more vasculature between cords that formed	160%
VEGFAxxxb antibody (5 ng/ml)	Swollen or perturbed cords, misshapen cords (Abcam, #ab14994)	Increased vasculature all throughout testis	195%
VEGFAxxxb antibody (50 ng/ml)	Swollen or perturbed cords, misshapen cords (Abcam, #ab14994)	Increased vasculature all throughout testis	195%

VEGFA = vascular endothelial growth factor A