Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis

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Abstract

Vascular endothelial growth factor-A (VEGFA) is a hypoxia-inducible peptide essential for angiogenesis and targets nonvascular cells in a variety of tissues and cell types. The objective of the current study was to determine the function of VEGF during testis development in bulls. We used an explant tissue culture and treatment approach to test the hypothesis that VEGFA-164 could regulate the biological activity of bovine germ cells. We demonstrate that VEGFA, KDR, and FLT1 proteins are expressed in germ and somatic cells in the bovine testis. Treatment of bovine testis tissue with VEGFA in vitro resulted in significantly more germ cells following 5 days of culture when compared with controls. Quantitative real-time RT-PCR analysis determined that VEGF treatment stimulated an intracellular response that prevents germ cell death in bovine testis tissue explants, as indicated by increased expression of BCL2 relative to BAX and decreased expression of BNIP3 at 3, 6, and 24 h during culture. Blocking VEGF activity in vitro using antisera against KDR and VEGF significantly reduced the number of germ cells in VEGF-treated testis tissue to control levels at 120 h. Testis grafting provided in vivo evidence that bovine testis tissue treated with VEGFA for 5 days in culture contained significantly more differentiating germ cells compared with controls. These findings support the conclusion that VEGF supports germ cell survival and sperm production in bulls.


Introduction

Spermatogenesis includes the proliferation, meiotic division, and morphological differentiation of germ cells within the seminiferous tubules of the testis leading to the production of spermatozoa. The regulation of germ cell differentiation is regulated by interactions with Sertoli cells (Russell & Griswold 1993) and extracellular signals including endocrine (Zirkin 1998) and locally produced factors (Parvinen & Ventela 1999). This process is initiated around puberty and results in sustained sperm production and potential fertility throughout the lifespan of an adult male. A key component of this process is the maintenance of the spermatogonial stem cell niche in the seminiferous tubule to provide differentiating spermatogonia (Oakberg 1971, McLean 2005).

Vascular endothelial growth factor (VEGF) is critical for angiogenesis, and its role in promoting new blood vessel growth has been extensively investigated (Hicklin & Ellis 2005). The VEGF family of proteins in mammals is encoded by five different genes: VEGFA, VEGFB, VEGFC, FIGF, and placenta growth factors (PGF-1 and PGF-2), of which VEGFA has the most profound effects on stimulating endothelial cell proliferation, survival, and differentiation (Hicklin & Ellis 2005). Alternative splicing of the VEGFA gene yields five different isoforms: VEGFA-120, VEGFA-144, VEGFA-164, VEGFA-188, and VEGFA-205; each differing in biological activity primarily associated with angiogenesis (Yamazaki & Morita 2006).

VEGFA isoforms stimulate endothelial and hematopoietic stem cells through two membrane-bound receptor tyrosine kinases, FLT1 (also known as VEGFR1) and KDR (also known as VEGFR2 and FLK1; Mac Gabhann & Popel 2005), which bind VEGFA with high affinity. Following the binding of VEGFA, each receptor elicits a distinct cellular response: FLT1 regulates cell–cell interactions (Takahashi & Shibuya 2005, Tammela et al. 2007), chemotaxis (Matsumoto et al. 2002), and cell survival (LeCouter et al. 2003), while KDR is responsible for the majority of signaling leading to proliferation, survival, and differentiation processes (Takahashi & Shibuya 2005, Tammela et al. 2007).

The importance of VEGFA, KDR, and FLT1 in biological processes such as embryogenesis (Carmeliet et al. 1996), folliculogenesis (Shimizu 2006), and seminiferous cord formation in the embryonic testis (Bott et al. 2006) has been demonstrated. An increasing number of reports suggest that VEGFA has direct effects on nonvascular cells including neuronal cells (Martí & Risau 1998, Wada et al. 2006, Nishijima et al. 2007), muscle cells (Germani et al. 2003, Bryan et al. 2008),...
and granulosa cells (Greenaway et al. 2004). Data also suggest potential functional roles for VEGFA in the postnatal testis, as KDR and FLT1 are expressed in testicular germ cells in humans (Ergun et al. 1997), rats (Rudolfsson et al. 2004), and mice (Nalbandian et al. 2003), while somatic Sertoli and Leydig cells produce VEGFA (Liu & Yang 2004). Two studies have shown that overexpression of VEGFA-120 and VEGFA-165 causes detrimental effects on male fertility in transgenic mice (Korpelainen et al. 1998, Huminiecki et al. 2001). Mutant mice lacking KDR or FLT1 have provided little insight into the role of VEGFA in the testis because embryonic death (E9–10) occurs before testis development (Ferrara et al. 1996, Korpelainen et al. 1998), and targeted inactivation of a single VEGF allele also results in embryonic death in utero (Ferrara et al. 1996). As a result, the role of VEGF in extravascular cells is poorly understood. Microarray analysis identified VEGFA as a candidate gene important for germ cell function in normal and grafted bovine testis in situ (Schmidt et al. 2007), and a study in our lab suggests that VEGFA may regulate germ cell proliferation in the bovine testis (Schmidt et al. 2006).

We hypothesize that VEGF is important for germ cell proliferation and/or survival in the developing bovine testis. To test this hypothesis, the objective of the current study was to determine the role of VEGFA-164, and its receptors, in regulating the activity of germ cells during the initiation of spermatogenesis in the bovine testis. To accomplish this objective, we used the experimental approach of treating bovine testis tissue in culture with VEGFA with three endpoints. First, we evaluated the kinetics of cell proliferation of somatic and germ cells in the seminiferous tubule of the cultured tissue. Secondly, we used quantitative analysis of the expression of genes important for germ cell proliferation and differentiation along with somatic cell–testis development to determine the specific cell mechanism regulated by VEGF. Finally, we surgically grafted the testis tissue treated with VEGFA in vitro to determine whether VEGF actions on germ cell function resulted in increased germ cells in the tissue several months after the VEGFA treatment. Based on our results, we conclude that VEGFA-164 is important for maintaining the survival of germ cells during the initiation of spermatogenesis in the bull testis. Thus, VEGFA-regulated germ cell survival is at least partially responsible for the eventual production of sperm in adult bulls.

**Results**

**Testicular expression of VEGFA and its receptors, KDR and FLT1**

To investigate what cell types express VEGFA, KDR, and FLT1 during bovine testis development in situ, immunohistochemical analysis was conducted on representative testis sections from 4- and 8-week-old bulls. Cytoplasmic staining for VEGFA was localized mainly in mitotically active spermatogonia (confirmed by proliferating cell nuclear antigen (PCNA) staining; Supplementary Figure 1a, which can be viewed online at www.reproduction-online.org/supplemental/) and, to a lesser extent, in the cytoplasm of Sertoli cells within seminiferous cords (Fig. 1A). In addition, endothelial cells and interstitial Leydig cells were immunopositive for VEGFA. KDR and FLT1 proteins were expressed in the germ, Sertoli, Leydig, and endothelial cells in the testes from both 4- and 8-week-old calves (Fig. 1B and C). The expression of VEGFA and receptors linked to its activity suggests that VEGFA may be important for germ and somatic cell proliferation, differentiation, and/or survival, which is independent of endothelial cell function. Mouse kidney was used as a positive control for the positive immunolocalization of these proteins (Fig. 1E). Primary antibodies were omitted as a negative control in bovine testis (Fig. 1D) and mouse kidney (Fig. 1F) respectively.

**Quantitative real-time RT-PCR analysis of genes regulated by VEGF in cultured bovine testis tissue**

To provide more insight regarding how VEGFA may regulate the activity of germ and somatic cells, testis tissue was cultured and treated with recombinant mouse VEGFA-164 (VEGF; 100 ng/ml) or vehicle. Mouse VEGFA-164 shares 88% amino acid sequence identity with corresponding regions of bovine VEGF-164. The expression of genes involved in cell survival, testis development, and VEGF signaling was assayed by quantitative real-time RT-PCR (qRT-PCR)
with gene-specific primer pairs (Table 1) at 0, 3, 6, and 24 h during culture and treatment. Data are presented as fold change (mean normalized expression values) relative to the 0 h (4-week-old donor) expression level.

KDR and FLT1 proteins are localized to germ and somatic cells in the bovine testis (Fig. 1B and C); thus, we assayed whether VEGF treatment would affect the expression of these genes during explant culture. In comparison with baseline expression (0 h), the expression of KDR and FLT1 was significantly induced at 3 h, regardless of treatment. In addition, VEGF treatment significantly increased (P<0.01) the expression of KDR and FLT1 mRNA at 24 h when compared with controls (Fig. 2A and B). It is known that VEGF binding leads to downregulation of KDR and FLT1 proteins at the cell surface as a means of receptor desensitization. However, it has been demonstrated that VEGF treatment upregulates the expression of KDR and FLT1 mRNA and proteins in several cell types (Barleon et al. 1997, Hervé et al. 2006, Yao et al. 2007), which is consistent with our observations in bovine testis tissue in vitro.

The BCL2 family of proteins functions as regulators of cell survival and comprises both anti-apoptotic (BCL2 and BCL2L1) and pro-apoptotic (BAX and BAD) members. Therefore, the ratio of mRNA expression between the pro-survival BCL2 and pro-apoptotic BAX was measured in control- and VEGF-treated bovine testis tissue explants. In respect to the 0-h time point, VEGF treatment significantly induced (P<0.001) the ratio of BCL2 to BAX expression in bovine testis tissue at 3 h (sixfold), 6 h (threefold), and 24 h (threefold) when compared with controls (Fig. 2C). VEGF can protect extravascular cells (Lambrechts et al. 2003, Jin et al. 2006) from hypoxia-induced cell death; therefore, we investigated the mRNA expression of pro-apoptotic BCL2/adenovirus E1B 19 kDa interacting protein (BNIP3), a hypoxia-responsive gene, following VEGF treatment. Regardless of treatment, BNIP3 mRNA was significantly higher at all time points during explant culture in comparison with the 0 h time point (Fig. 2D).

<table>
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<tr>
<th>Gene</th>
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<th>Reverse sequence (5′−3′)</th>
<th>Amplicon size</th>
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Table 1 Bovine primer sequences used for quantitative real-time RT-PCR.

However, BNIP3 mRNA transcript levels were significantly suppressed at 3 h (P<0.01), 6 h, and 24 h (P<0.001) respectively after VEGF treatment compared with vehicle-treated testis tissue culture controls (Fig. 2D). These data demonstrate that VEGFA stimulates an intracellular response that prevents germ cell death.

BNIP3 is a major downstream effector of hypoxia-inducible factor 1, α-subunit (HIF1A) protein (Althaus et al. 2006); therefore, the expression of HIF1A was evaluated in bovine testis tissue during the explant culture period. Consistent with the qRT-PCR analysis for BNIP3 expression, HIF1A transcript levels were

Figure 2 Expression of genes important for cell survival, testis development, and VEGF signaling. Testis tissue explants from 4-week-old donors were cultured with VEGF (100 ng/ml) or vehicle alone. Briefly, samples obtained at 3, 6, and 24 h following treatment were assayed for gene expression by qRT-PCR for (A) KDR, (B) FLT1, (C) ratio of BCL2:BAX, (D) BNIP3, (E) HIF1A, (F) PTGS2, (G) KIT, and (H) GATA4. Results were normalized to RPS2 expression, standardized to baseline expression at 0 h, and are presented as the fold change of mean expression values ± S.E.M. Asterisks indicate significant differences between control and treatment means at a given time point. ***P<0.001; **P<0.01; and *P<0.05. Results are representative of independent experiments for each time point (n≥3), each performed on duplicate samples.
significantly reduced in VEGF-treated bovine testis tissue at 3 h (P<0.001) and 6 h (P<0.05) when compared with controls (Fig. 2E). Prostaglandin-endoperoxide synthase 2 (PTGS2) is critical for germ cell maturation and somatic–germ cell interactions in the mammalian gonad both in vivo and in vitro (Neal et al. 1975, Takahashi et al. 2006, Winnall et al. 2007). Recent evidence suggests that this multifunctional enzyme is also responsible for protecting mouse embryonic stem cells and renal interstitial cells from oxidative stress and hypertonicity-induced apoptosis (Moeckel et al. 2003, Liou et al. 2007); thus, we evaluated the mRNA expression of PTGS2 in response to VEGF treatment. In respect to the 0-h time point, VEGF treatment significantly induced the expression of PTGS2 in bovine testis tissue at 3 h, 6 h (P<0.001), and 24 h (P<0.01) when compared with controls (Fig. 2F). Therefore, VEGF treatment promoted several independent means for cell survival in bovine testis tissue.

The mRNA expression of the testis germ cell-specific gene KIT and Sertoli and Leydig cells expressed gene GATA4 were analyzed in explant-cultured bovine testis tissue treated with VEGF (100 ng/ml) or vehicle. In comparison with the 0-h time point, KIT expression was significantly higher (P<0.05) in the VEGF treatment group at 3 and 6 h respectively, while control levels were not different from baseline levels at 3, 6, or 24 h. However, no differences in KIT were detected between control and treatment groups at 3, 6, and 24 h. GATA4 expression was twofold lower (P<0.05) in control- and VEGF-treated testis tissue at all time points when compared with the 0-h baseline. Regardless of treatment, KIT and GATA4 mRNA expression was not different between control- and VEGF-treated testis tissues at 3, 6, and 24 h during culture (Fig. 2G and H).

**Cell types affected by VEGF treatment in cultured bovine testis tissue**

The specificity of the VEGF treatment effect to stimulate cell survival pathways was investigated to determine the testicular cell type responding to the VEGF signal. We obtained testis tissue from 4-week-old donors and treated the tissue with vehicle or 100 ng/ml VEGFA during explant culture. In addition, a negative control was added to investigate the specificity of the VEGF signal during explant culture by supplementing media with neutralizing antibodies against human KDR (3 mg/ml) and mouse VEGF (1.5 mg/ml) at concentrations necessary to yield one-half maximal inhibition (ND50) of VEGF activity from treatment (100 ng/ml). Because KDR and FLT1 receptors are present on germ and Sertoli cells (Fig. 1), we investigated the population of these cells during (72 h) and following (120 h) the explant culture period by immunostaining for known markers of germ (DDX4/MVH) and Sertoli cells (GATA4). PCNA immunostaining indicated no differences in the rate of germ cell proliferation between control- and VEGF-treated bovine testis tissue grafts (data not shown). Germ cell numbers were 30% greater (P<0.05) in bovine testis tissue treated with VEGF when compared with controls at 120-h of culture (Fig. 3A). Blocking VEGF activity using antisera against KDR and VEGF significantly reduced (P<0.05) the number of germ cells in VEGF-treated testis tissue to control levels at 120 h. The effect of VEGF treatment on germ cell numbers was decreased by 21% when neutralizing the activity of KDR. Directly mitigating the ligand activity in VEGF-treated testis tissue reduced germ cell numbers by 52%. Immunostaining for DDX4 to demonstrate the presence of germ cells in cultured tissues is shown in Fig. 3B. In contrast, Sertoli cell number did not change during the explant culture period and was unaffected by treatment.

**Figure 3** Effect of VEGF treatment on survival of specific cell types in bovine testis tissue. (A) Analysis of germ and Sertoli cell populations in control- and VEGF-treated testis tissue following explant culture and blockade of VEGF activity. (B) Morphometric analysis of germ cell number was facilitated using DDX4/MVH immunostaining. An arrow indicates the presence of a DDX4-positive germ cell. Data are presented as the mean ± s.e.m. Different letters indicate significant differences between means (P<0.05). Scale bar, 50 μm.
or blockage of VEGF biological activity (Fig. 3A). Collectively, these data indicate that VEGF treatment promotes germ cell survival and results in increased germ cells following 5 days of explant culture.

**Testicular tissue explant culture followed by xenografting bioassay**

The testis tissue culture results demonstrated that VEGF supports germ cell survival in the bovine testis. The next step in the project was to determine whether increased germ cell survival following VEGF treatment *in vitro* resulted in long-term increased production of sperm in testis tissue. To complete this objective, treatment of testis tissue *in vitro* with VEGF was combined with testis tissue grafting to determine the extent of spermatid production that resulted from increased spermatogonia survival. Testicular tissue obtained from bovine donors was cultured and treated with VEGF at 0 (vehicle), 100, or 200 ng/ml as tissue explants for 5 days then grafted onto nude mice. Bovine testis tissue grafts were removed from mice when testis tissue age equaled 32 weeks and categorized as functional based on evidence of germ cell differentiation in the seminiferous tubules of the testis graft. No differences existed between donor age (4 weeks versus 8 weeks) and grafting period, so these datasets were pooled. Following explant culture, no gross morphological differences were observed due to treatment (Fig. 4A–C). Immunostaining with von Willebrand factor (VWF) indicated no differences in blood vessel number or microvascular density (MVD) between control- and VEGF-treated testis tissue (Fig. 4D).

Treatment of testis tissue for 5 days in culture with VEGF or vehicle did not change the number of functional grafts recovered after grafting these tissues (Supplementary Figure 1b). In addition, no differences were observed in graft weight or seminiferous tubule cross-section number in the recovered grafted tissue between the tissue groups treated *in vitro* with VEGF or vehicle (Fig. 4E and F). The long-term ability of Leydig cells to produce testosterone was unaffected, as no difference in serum testosterone was detected by RIA in mice grafted with testis tissue treated with VEGF or vehicle during explant culture (Fig. 4G). Similarly, the analysis of vesicular gland weights in recipient mice confirmed that testosterone was biologically active (data not shown).

To determine whether treating bovine testis tissue with VEGF for 5 days in culture had a long-term effect on sperm production, we analyzed the germ cell populations in functional testis grafts. Regardless of treatment, all testis xenografts contained spermatogonia and meiotic germ cells when removed from recipient mice. However, bovine testis tissue treated with VEGF in culture had significantly more (*P* < 0.005) differentiating germ cells after the grafting period compared with control tissue grafts (Fig. 5A). VEGF treatment of cultured testis tissue for 5 days with 100 ng/ml resulted in 36% of testis grafts supporting the completion of meiosis (determined by the presence of haploid spermatids) when compared with only 4.8% of grafts in the control group. Similarly, 40.9% of grafts in the 200 ng/ml VEGF treatment group contained spermatids. No dose effect (100 vs 200 ng/ml) on germ cell differentiation was detected (*P* = 0.63). Thus, treatment of cultured testis tissue with VEGF at 100 and 200 ng/ml resulted in significantly more spermatid production than controls. A light micrograph demonstrating active spermatogenesis in a bovine testis tissue graft is shown in Fig. 5B. Seminiferous tubule cross sections containing spermatogonia within grafts were classified based on the existence of meiotic germ cells (primary and/or secondary spermatocytes) and haploid spermatids (round, elongating, and/or elongate spermatids). In addition to cell identification based on morphological characteristics, immunostaining for synaptonemal complex protein 3 (SYCP3), a protein marker of meiosis (Parra *et al.* 2004), confirmed the presence of

![Figure 4](image-url)
spermatocytes in bovine testis tissue grafts (Supplementary Figure 1c and d). When compared with controls, seminiferous tubules of bovine testis grafts cultured with VEGF contained significantly more (P<0.05) meiotic germ cells and spermatids (Fig. 5C and D). Thus, short-term treatment of bovine testis tissue with VEGF prior to grafting results in significantly more spermatids present in the tissue 24 and 28 weeks later.

Seminiferous tubules of each testis graft were evaluated for numbers of spermatogonia to determine the effect of VEGF treatment on long-term germ cell survival in cultured bovine testis tissues. Testis tissue cultured and treated with VEGF prior to grafting contained significantly more (P<0.001) spermatogonia than vehicle alone when removed from recipient mice (Fig. 5E). VEGF treatment at 100 and 200 ng/ml resulted in more seminiferous tubules containing spermatogonia (78.8 and 88.1% respectively) in testis grafts in comparison with controls (55.2%). The inverse relationship was observed for the proportion of seminiferous tubule cross sections containing only Sertoli cells (Fig. 5F). Hence, germ cell loss was significantly reduced (P<0.001) in bovine testis tissue treated with VEGF in culture prior to ectopic grafting. These data provide in vivo evidence that VEGF support of spermatogonia survival leads to the production of more sperm.

**Discussion**

The purpose of this research was to investigate the role of VEGF in the regulation of germ cell survival and differentiation; two processes essential for sperm production. Work in our laboratory demonstrated that increased production of differentiating germ cells in bovine testis tissue ectopic grafts treated with a single dose of VEGF directly on the tissue at the time of grafting (Schmidt et al. 2006). However, the specific target or effect of VEGF treatment was not determined. Owing to the complex nature of somatic and germ cells interaction in the testis required for germ cell differentiation, alteration of activity of several cell types may lead to changes in germ cell production. VEGFA, KDR, and FLT1 protein expression was localized mainly to proliferating germ cells, Sertoli cells, and Leydig cells in the testis of 4- and 8-week-old bull calves. VEGF treatment significantly increased the population of germ cells in testis tissue cultures when compared with controls. No changes in the somatic cells were observed in these experiments leading us to conclude that VEGF acts to stimulate functional mechanisms in undifferentiated germ cells during postnatal bovine testis development to support the proliferation and/or survival of these cells.

To test our hypothesis that VEGF promotes germ cell survival, we conducted qRT-PCR to evaluate expression of genes important for cellular survival, specifically the balance between anti-apoptotic and pro-apoptotic proteins that have been associated with VEGF action in other cell types. Therefore, we assayed the ratio of BCL2: BAX expression and found that VEGF treatment significantly up-regulated the mRNA expression of anti-apoptotic BCL2 relative to pro-apoptotic BAX when compared with controls at 3, 6, and 24 h of culture. Since VEGF has been implicated in protecting extra-vascular cells (Lambrechts et al. 2003, Jin et al. 2006) from hypoxia-induced cell death, we also investigated the mRNA expression of pro-apoptotic BNIP3, a hypoxia-responsive gene, during explant culture. VEGF treatment suppressed the expression of BNIP3 mRNA at all time points during explant culture when compared with controls, and this correlates with HIF1A transcripts levels (Fig. 2C). In contrast, VEGF treatment did not influence GATA4 mRNA levels in the testis tissue explants culture, supporting our conclusion that VEGF does not regulate Sertoli and Leydig activity in the bovine testis. Similarly, VEGF treatment did not regulate the expression of KIT, a gene associated with germ cell differentiation in the testis, when compared with controls. This indicates that VEGF regulates undifferentiated germ cells during explant culture.
VEGF signaling is mediated through the transmembrane receptor tyrosine kinases, KDR and FLT1. VEGF treatment significantly induced the expression of its receptors KDR and FLT1 at 24 h when compared with controls in bovine testis tissue cultures (Fig. 2G and H). In normal and neoplastic cells, KDR activation rapidly induces several signaling cascades including phospha-
tidylinositol 3-kinase, phospholipase C-γ, MAPK, and Src involved in promoting cellular proliferation, survival, and differentiation events (Takahashi & Shibuya 2005, Yamazaki & Morita 2006, Tamnella et al. 2007). Activation of FLT1 elicits a poor mitogenic response (in contrast to KDR) and primarily functions to regulate chemotaxis, survival, and cell–cell interactions in a variety of cell types (Kaplan et al. 2005, Takahashi & Shibuya 2005, Fragoso et al. 2006). It is also established that ligand binding to FLT1 directly attenuates down-
stream-signaling events mediated by KDR. The expression of VEGF, KDR, and FLT1 in somatic and germ cells of the testis suggests this group of molecules may support spermatogenesis in several distinct ways. Indeed, signaling pathways stimulated by VEGF have been implicated in germ cell homeostasis (Dolci et al. 2001), and several studies have demonstrated the importance of VEGF for male fertility (Korpelainen et al. 1998, Humniecki et al. 2001, Bott et al. 2006).

Gene expression of VEGF is regulated by a variety of stimuli such as hypoxia, growth factors, estrogen, and gonadotropins, and we demonstrate overlapping expression of VEGF and its receptors in germ and somatic cells in the pre-pubertal bovine testis, similar to the expression pattern found in the embryonic mouse testis (Bott et al. 2006). Based on the data presented here, we hypothesize that proliferating undifferentiated germ cells may produce VEGF in response to local hypoxia and oxidative stress to prevent apoptosis in these cells preceding the onset of puberty. Accordingly, testicular germ cells of several species express KDR and FLT1 (Ergun et al. 1997, Nalbandian et al. 2003, Rudolffson et al. 2004), and although these receptors are expressed in endothelial cells, no active angiogenesis takes place within the postnatal testis. VEGF synthesis by Leydig cells is stimulated in response to hCG/LH (Rudolffson et al. 2004), and FSH triggers Sertoli cell production of VEGF in the adult testis (Marti & Risau 1998, Liu & Yang 2004, Reisinger et al. 2007). Interestingly, FSH has been shown to stimulate proliferation of spermatogonia (De Rooij et al. 1989), and type A spermatogonia express KDR in neonatal mice (Nalbandian et al. 2003). Thus, autocrine and paracrine regulation of germ cell activity by VEGF in the mammalian testis during early development and adulthood may be important for the initiation and maintenance of sperm production.

Therefore, we sought to determine whether the changes induced by VEGF in vitro in germ cells lead to increased production of sperm in the tissue, on a long-
term basis. To complete this objective, we grafted testis tissue explants after culture and treatment with VEGF or vehicle onto recipient nude mice and evaluated germ cell differentiation and sperm production in the grafts at recovery, 6 months later. While we have cultured and grafted bovine testis tissue successfully, no researchers have treated tissue in vitro prior to grafting to determine whether the treatment modifies the germ cell population such that long-term sperm production is affected. Our testis tissue culture-grafting approach is a novel method to investigate factors important for germ cell biology because 1) sperm production in testis tissue grafts is a direct means to determine whether a specific treatment manipulates the germ cell population and 2) the specialized microenvironments (Honaramooz et al. 2002, McLean 2005, Hess et al. 2006, Yoshida et al. 2007) established between germ and Sertoli cells are maintained.

Treating bovine testis tissue explants with VEGF in culture prior to grafting did not affect growth, recovery rate, or testosterone synthesis by the grafted testis tissue when compared with controls. The rate of testis tissue graft growth in this study was consistent with previous explant culture experiments, and the number of seminiferous tubule cross sections within testis grafts treated with VEGF or vehicle alone did not differ (Schmidt et al. 2006). Interestingly, a single VEGF treatment of bovine testis tissue at the time of grafting resulted in significantly larger grafts 32 weeks later compared with controls, despite the fact that the number of seminiferous tubules was not different between groups (Schmidt et al. 2006). Although the exact reason for the larger graft size has not been determined and an increase in blood vessel growth was not detected, it is possible that VEGF stimulated cell growth outside of the seminiferous tubules. Thus, our current approach aimed to minimize the effect of VEGF on neovascularization within the graft site of the recipient mouse.

Regardless of treatment, all bovine testis tissue grafts contained spermatogonia and meiotic germ cells. However, culturing bovine testis explants with VEGF resulted in greater than sevenfold increase (P<0.05) in the extent of germ cell differentiation, as a greater number of testis grafts producing meiotic germ cells and spermatids. Morphometric evaluation of seminiferous tubule cross sections containing only Sertoli cells suggests that VEGF treatment significantly increased (P<0.001) germ cell survival in testis tissue grafts. Abrogating the biological activity of VEGF during explant culture with neutralizing antibodies confirmed these findings in vitro. No differences in Sertoli cell number were observed in either study. Regardless of treatment, testis tissue grafts produced similar levels of testosterone, so we conclude Leydig cell function was not affected.

Binding of VEGF to its cognate receptors initiates a variety of signaling cascades similar to those stimulated by GDNF and KIT in the postnatal testis, two factors of known importance for regulating germ cell proliferation,
survival, and differentiation (Meng et al. 2000, Ohta et al. 2000, 2003, Rossi et al. 2000, Dolci et al. 2001, Aponte et al. 2006, Naughton et al. 2006, Payne & Braun 2007). Rodent studies have shown that male fetal germ cells express VEGF protein until shortly after birth (Bott et al. 2006), and Sertoli cells continue to produce VEGF throughout adult life (Marti & Risau 1998, Nalbandian et al. 2003). Interestingly, type A spermatogonia in close association with Sertoli cells also express KDR but do not produce VEGF in mice aged 5 days post partum and older. This developmental switch within the specialized microenvironment of the seminiferous tubule suggests that VEGF may be important in regulating both the onset and maintenance of spermatogenesis. Collectively, these data taken together with our recent observations in bovine testis advocate the importance of VEGF for male fertility and support our hypothesis that VEGFA-164 maintains the survival of germ cells during the initiation of spermatogenesis in the developing bovine testis.

Materials and Methods

Chemicals and reagents

All reagents were purchased from Sigma unless stated.

Donor bulls and tissue collection

The Washington State University Animal Care and Use Committee approved all animal procedures. Testis tissue samples were obtained from 4- and 8-week-old bull calves (ten bulls/age) at the Washington State University Beef Center using standard castration protocols. Testes were immediately processed for explant culture and histological examination, as previously described (Schmidt et al. 2006).

Immunohistochemistry

Immunohistochemistry was performed, as previously described (Caires et al. 2008), using the avidin–biotin complex method with affinity-purified primary antibodies (diluted in 10% normal serum, pH 7.4) as described in Table 2. Immunoreactivity was detected following a 5-min incubation in 3,3′-diaminobenzidine, and sections were counterstained with hematoxylin. As a negative control, serial sections were processed without primary antibody.

Testicular tissue explant culture

Testicular parenchyma (~3 mg) obtained from 4- and 8-week-old bovine donors were cultured as tissue explants on 0.45-µm pore filtering filter membranes (Millipore, Bedford, MA, USA) for 5 days prior to grafting. Eight tissue explants per donor (n=13) were cultured at 32 ºC in DMEM containing 10% fetal bovine serum, 30 mg/ml penicillin, and 50 mg/ml streptomycin, including recombinant mouse VEGFA-164 (493-MV; R&D Systems, Minneapolis, MN, USA) at 0 (control), 100, or 200 ng/ml. Media were changed every 2 days including fresh addition of VEGF, and each experiment was conducted in duplicate. Following explant culture, tissues were processed for evaluation of angiogenesis using an established method (Vermeulen et al. 1996). Briefly, blood vessels were identified by tubular morphology (single layer of endothelial cells and presence of intraluminal red blood cells) and positive immunostaining for VWF (1:400). MVD was then calculated by enumerating the mean number of blood vessels per 40× field in ten randomly chosen areas within testis tissue from each treatment group (n=5), as previously reported (Sinha et al. 2004).

Quantitative real-time RT-PCR analysis

Bovine testis tissue (from 4-week-old donors) explants treated with vehicle (0 ng/ml) or VEGF (100 ng/ml) were randomly selected at 0, 3, 6, and 24 h during culture and homogenized in TRIzol (Invitrogen) according to the manufacturer’s protocol. Total RNA was purified, dissolved in RNase-free water (Ambion, Foster City, CA, USA), and 2 µg total RNA was reverse transcribed into cDNA using an oligo(dT) 12–18 primer and M-MLV reverse transcriptase (Invitrogen). cDNA synthesis was confirmed using primers and PCR for b-actin. Gene expression was evaluated in control- and VEGF-treated samples at each time point (n≥3 donors). Primers were

Table 2 Antibodies, suppliers, and dilutions used for immunohistochemical analysis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody description</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX4/VASA</td>
<td>Rabbit anti-human DDX4/MHV polyclonal antibody</td>
<td>Abcam, Cambridge, MA, USA</td>
<td>1:500</td>
</tr>
<tr>
<td>GATA4</td>
<td>Rabbit anti-mouse GATA4 polyclonal antibody</td>
<td>SCBT, Santa Cruz, CA, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>PCNA</td>
<td>Mouse monoclonal PCNA antibody (PC10)</td>
<td>DAKO, Carpinteria, CA, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>SYCP3-C</td>
<td>Rabbit anti-human SYCP3 polyclonal antibody</td>
<td>Novus, Littleton, CO, USA</td>
<td>1:400</td>
</tr>
<tr>
<td>SYCP3-N</td>
<td>Rabbit anti-mouse SYCP3 polyclonal antibody</td>
<td>Novus</td>
<td>1:400</td>
</tr>
<tr>
<td>VEGF</td>
<td>Rabbit anti-mouse VEGF polyclonal antibody</td>
<td>SCBT</td>
<td>1:400</td>
</tr>
<tr>
<td>VEGFR1/FLT1</td>
<td>Rabbit anti-mouse FLT1 polyclonal antibody</td>
<td>SCBT</td>
<td>1:200</td>
</tr>
<tr>
<td>VEGFR2/KDR</td>
<td>Rabbit anti-mouse FLK1 monoclonal antibody</td>
<td>SCBT</td>
<td>1:200</td>
</tr>
<tr>
<td>VWF</td>
<td>Goat anti-human von Willebrand factor (C-20)</td>
<td>SCBT</td>
<td>1:400</td>
</tr>
</tbody>
</table>

ABCAM, Abcam, Inc.; DAKO, Dako Corporation; Novus, Novus Biologicals; SCBT, Santa Cruz Biotechnology.
Neutralization of VEGF bioactivity

All antisera were obtained from R&D Systems. Bovine testis tissue from 4-week-old donors \( (n=5) \) was cultured as explants (as described above) in the presence of VEGF-A164 at 0 (control) and 100 ng/ml. Neutralizing antibodies including a goat anti-human KDR polyclonal antiserum (AF-357, 3 mg/ml) and a goat anti-mouse VEGF polyclonal antiserum (AF-493-NA, 1.5 mg/ml) were added to media at concentrations to yield one-half maximal inhibition (ND\(_{50}\)) of VEGF-A164 activity due to treatment (100 ng/ml). We evaluated germ cell and Sertoli cell populations in cultured explants by counting the mean number of each cell type found within ten randomly selected seminiferous tubule cross sections in testis tissue \( (n=4; \) for each treatment group) at 72 and 120 h respectively.

Ectopic testis tissue xenografting bioassay

After 5 days in culture and \textit{in vitro} treatment, testis tissues were ectopically grafted onto castrated immunodeficient NCr nude mice (Taconic, Hudson, NY, USA; CrTac:NCR-Fox1 <nu>), as previously described (Oatley \textit{et al.} 2005). Recipient mice were killed when grafted bovine testis reached a tissue age of 32 weeks, serum was collected, and recovered tissues were processed for histological evaluation. The extent of germ cell survival and differentiation was based on the presence of spermatogonia, spermatocytes, and spermatids in testis grafts according to morphological characteristics, as previously described (Caires \textit{et al.} 2008).

Statistical analysis

All datasets were presented as the mean \( \pm \text{S.E.M.} \), and differences between treatments were considered significant at \( P<0.05 \). Parametric data were analyzed using ANOVA, and pairwise comparisons between groups were conducted using the Duncan’s multiple range test for significance. Nonparametric datasets were evaluated using the Kruskal–Wallis test, and comparisons between groups were conducted using Mann–Whitney tests.

Declaration of interest

There is no conflict of interest prejudicing the impartiality of this research.

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