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Vascular Endothelial Growth Factor and Kinase Domain Region Receptor Are Involved in Both Seminiferous Cord Formation and Vascular Development During Testis Morphogenesis in the Rat

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Abstract

Morphological male sex determination is dependent on migration of endothelial and preperitubular cells from the adjacent mesonephros into the developing testis. Our hypothesis is that VEGFA and its receptor KDR are necessary for both testicular cord formation and neovascularization. The *Vegfa* gene has 8 exons with many splice variants. *Vegfa120*, *Vegfa164*, and *Vegfa188* mRNA isoforms were detected on Embryonic Day (E) 13.5 (plug date = E0) in the rat. *Vegfa120*, *Vegfa144*, *Vegfa164*, *Vegfa188*, and *Vegfa205* mRNA were detected at E18 and Postnatal Day 3 (P3). *Kdr* mRNA was present on E13.5, whereas Fms-like tyrosine kinase 1 receptor (*Flt1*) mRNA was not detected until E18. VEGFA protein was localized to Sertoli cells at cord formation and KDR to germ and interstitial cells. The VEGFA signaling inhibitors SU1498 (40 μM) and VEGFR-TKI (8 μM) inhibited cord formation in E13 testis cultures with 90% reduced vascular density ($P < 0.01$) in VEGFR-TKI-treated organs. Furthermore, Je-11 (10 μM), an antagonist to VEGFA, also perturbed cord formation and inhibited vascular density by more than 50% ($P < 0.01$). To determine signal transduction pathways involved in VEGFA's regulation of testis morphogenesis, E13 testis were treated with LY 294002 (15 μM), a phosphoinositide 3-kinase (PI3K) pathway inhibitor, resulting in inhibition of both vascular density (46%) and cord formation. Thus, we support our hypothesis and conclude that VEGFA, secreted by the Sertoli cell, is involved in both neovascularization and cord formation and potentially acts through the PI3K pathway during testis morphogenesis to elicit its effects.

Keywords

growth factors; Sertoli cells; testis

INTRODUCTION

The formation of the seminiferous cords occurs on Embryonic Day (E) 13.5 in the rat (plug date = E0) and is the first morphological indicator of testis differentiation [1,2]. *Sry* gene expression by the Sertoli cell [3] induces the expression of other Sertoli cell-specific genes that cause Sertoli-primordial germ cell aggregation and proliferation of Sertoli cells within the indifferent testis [4,5]. Following this cellular aggregation, mesenchymal preperitubular cells and endothelial cells migrate from the adjacent mesonephros into the differentiating testis to envelop the Sertoli-germ cell aggregates [6,7]. The cell types that migrate from the mesonephros into the testis are endothelial and preperitubular in origin [8]. It is not known

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how these mesonephric cells are induced to migrate into the testis, if they are pluripotent cells that differentiate into multiple cell types, or if they are independent cell types that migrate due to single or multiple growth factor signal transduction pathways [9,10]. Recent evidence suggests that endothelial cells are potential precursors of Leydig cells [11], and granulosa cells have many endothelial-like properties [12,13]. Therefore, it is possible that pre-endothelial cells are necessary for the development of many different cell types within the gonads, in addition to cell types comprising vasculature.

Removal of the mesonephros before mesonephric cell migration precludes cord formation. Therefore, mesonephric cell migration appears to be crucial for testis development [6,7,14]. Mesonephric cells migrated through ovaries toward testis explants in mesonephric-ovary-testis sandwich cultures [8], supporting the concept that a cell type in the testis induces mesonephric cell migration. Because germ cell deficient mice have normal cord formation [15-17], the Sertoli cells logically produce the paracrine growth factor(s) that induce mesonephric cell migration [15-18]. Several factors, including the activity of Sertoli-derived chemokines, have been proposed, because of alterations in seminiferous cord formation in vitro [19-23] and altered testis morphogenesis in vivo [24,25].

At gonadal differentiation, another morphological event that occurs is development of sex-specific vasculature [5,9]. Presently, it is unknown whether the testis vasculature develops at the time of seminiferous cord formation or after testis morphogenesis. However, because both pre-endothelial cells and pre-peritubular cells migrate into the differentiating testis as gonadal morphogenesis is initiated [8], we hypothesized that both seminiferous cord formation and vasculogenesis in the testis occur simultaneously. To test this hypothesis we chose to evaluate whether VEGFA, because of its involvement in establishment of vasculature within other organ systems, regulated testis morphogenesis.

The *Vegf* family is encoded by 5 different genes: *Vegfa (Vegf), Vegfb, Vegfc, Vegfd*, and placenta growth factor. VEGFA (VEGF) is the most potent growth factor involved in neovascularization of tissues and tumors. VEGFA acts through two receptors: FLT1 (also known as VEGFR1) and KDR (also known as VEGFR2). Interaction between VEGFA and KDR promotes the development of new vasculature in developing organs, whereas VEGFA binding to FLT1 maintains and enhances survival of endothelial cells [26].

The *Vegfa* gene can undergo differential splicing to form many different isoforms. Five isoforms are well documented (*Vegfa205*, *Vegfa188*, *Vegfa164*, *Vegfa144*, and *Vegfa120*), whereas three of the isoforms (*Vegfa188*, *Vegfa164*, and *Vegfa120*) have been determined to be expressed within most tissues of the body [27]. The function of VEGFA during embryonic development is critical, because even the loss of one allele results in embryonic lethality before testis development in transgenic mice [28,29]. Homozygous knockouts for either FLT1 or KDR die on E9-10 before testis development [30,31], thereby preventing their use as a model in these studies. Overexpression of VEGFA in transgenic mice is also detrimental to fertility and embryonic viability [32,33]

The role of VEGFA and its receptors during embryonic testis morphogenesis has not been investigated, nor has VEGFA been linked to regulation of events associated with cord formation and sex-specific development of vasculature during testis morphogenesis. Therefore, the objectives of the current study were to determine whether the angiogenic factor, VEGFA, and its receptor, KDR, were important in testis morphogenesis.

MATERIALS AND METHODS

RNA Isolation and Reverse Transcription of *Vegfa* Isoforms, and *Kdr* and *Flt1* Receptor mRNA

Total RNA was obtained from gonadal tissue of various developmental stages by using Tri Reagent as per the manufacturer's protocol (Sigma, St. Louis, MO). After isolation, total RNA was resuspended to a volume of 20 μl, and 2.5 μl of this was reverse transcribed using SuperScript II (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol [34]. The resulting cDNA was then stored at -20°C.

Primers

Primers for rat VEGFA were used to generate different PCR fragments, depending on the *Vegfa* isoform expressed, namely a 431-bp product for *Vegfa120*, 500 bp for *Vegfa144*, 563 bp for *Vegfa164*, 635 bp for *Vegfa188*, and 750 bp for *Vegfa205* [35]. Conventional PCR was performed with *Vegfa* primers 5′-ctgctctcttgggtgcactg-3′ and 5′-caccgccttggcttgtcacat-3′ at an annealing temperature of 58°C for 35 cycles. Human specific primers were used to amplify a 324-bp product for *Flt1* (5′-caggctcatgaacttgaaagc-3′ and 5′-gaaggcatgaggatgagagc-3′) and a 213-bp product for *Kdr* (5′-caaaaattgtttctggggc-3′ and 5′-cagcttccaagcggctaagg-3′) [36] at an annealing temperature of 56°C for 30 cycles for both sets of primers. Primers for rat *Gapdh*, a constitutively expressed gene, were used to produce a 460-bp PCR fragment as a control for RNA isolation and amplification [37]. Conventional PCR was performed with *Gapdh* primers 5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′ at an annealing temperature of 58°C for 40 cycles. All PCR products were subcloned and confirmed using restriction digest analysis. PCR products were subcloned into pCRII (Invitrogen Life Technologies) with the TOPO TA Cloning kit (Invitrogen Life Technologies) and sequenced using primers provided with the kit (data not shown).

Embedding, Histologic Analysis, and Immunohistochemistry

Tissues were fixed in Bouin's solution and embedded in paraffin according to standard procedures [34]. The tissues were sectioned (thickness, 5 μm), deparaffinized, rehydrated, and microwaved in 0.01 M of sodium citrate for 5 min. After microwave treatment, tissues were cooled for 1-2 h and rinsed in 3% hydrogen peroxide in methanol for 20 min. Sections were then blocked with 10% normal goat serum in PBS for 30 min at room temperature. Immunohistochemistry was performed as described previously [38]. The KDR antibody was a mouse monoclonal IgG_1 antibody (Santa Cruz Biotechnology [SCB], Santa Cruz, CA) raised against a recombinant protein corresponding to amino acids 1158-1345 of KDR. The VEGFA antibody was an affinity purified rabbit polyclonal antibody (SCB) raised against a peptide corresponding to amino acids 1-140 of human VEGFA. Both antibodies were diluted 1:50-1:100 in 10% normal goat serum. As a negative control, serial sections were processed without any primary antibody. The biotinylated goat anti-rabbit and goat anti-mouse secondary antibodies were diluted 1:300. The secondary antibody was detected using aminoethyl carbazole (AEC) chromagen substrate solution (ZYMED Laboratories, San Francisco, CA). Sections were counterstained with hematoxylin to determine cell types that were stained with respective antibodies.

Organ Cultures

Timed pregnant Sprague-Dawley rats were obtained from our breeding colony at the University of Nebraska-Lincoln Animal Science Department, with founders being purchased from Charles River Laboratories (Wilmington, MA). All animal procedures were approved by the University of Nebraska Institutional Animal Care and Use Committee. The plug date was considered to be E0. E13 gonads were dissected with the mesonephros. Only embryos with tail somite (ts)

numbers between 15 and 18 were used for these experiments. One organ from each animal was designated as a vehicle control, and its pair was subjected to VEGFA receptor signal transduction antagonist, VEGFA antagonist treatment, or PI3K-inhibitor treatment. The organs were cultured in drops of media on Millicell-CM filters (Millipore, Bedford, MA) floating on the surface of 0.4 ml of CMRL Medium-1066 (Gibco, Grand Island, NY) supplemented with penicillin-streptomycin, insulin (10 μg/ml), transferrin (10 μg/ml), L-glutamine (52 μg/ml), and BSA (0.1%). Approximately 14-20 testis organ pairs were exposed daily to each of four inhibitors at the following doses: VEGFR-TKI (VEGFA receptor antagonist [cat no. 676475]), 4 and 8 μM; SU1498 (KDR receptor antagonist), 20 and 40 μM; Je-11 (VEGFA antagonist), 10 μM; or 10 μM or 15 μM of the PI3K inhibitor LY 294002 (Calbiochem, La Jolla, CA). Each inhibitor was added directly to the culture media while DMSO was added at a similar dose as the treatment to paired vehicle controls. Media was changed once after 2 days of culture. Organs were cultured for 3 days, by which point cords had developed in the controls. Only those organ pairs whose control formed cords were used for analysis. We also determined that viability was retained in our culture systems at each dose of inhibitor, because of no alterations in labeling of mitotic germ cells for PECAM, as measured by the confocal microscope. The only differences we detected in PECAM staining were in vasculature surrounding germ-Sertoli cell aggregates. The concentration required for 50% inhibition (IC50) of VEGFR-TKI was 2.0 μM for FLT1 and 0.1 μM for KDR, as detected in cell cultures [39]. Because organ cultures have 100 more cell layers than a cell culture system, our use of 4 and 8 uM was within reasonable doses. The inhibitory strength of all other inhibitors used was less than the IC50 for cell cultures. A higher dose of Je-11 could not be used because the levels of DMSO necessary in the final preparation of the compound would have impaired testis morphogenesis in our controls.

Imaging and Area Analysis of Organ Cultures

All male organs, as confirmed by PCR for *Sry* expression in tail samples from each embryo [20], were imaged before processing for whole-mount immunohistochemistry to verify the presence of seminiferous cords in control organs and to analyze testis area. Brightfield images of whole organs from the VEGFR-TKI and Je-11 treatments were obtained with a Spot camera and Spot imaging capture system (Diagnostic Instruments; Spot Advance, Sterling Heights, MI). Pixel areas were quantified using the NIH Scion image program (Scion Image, Frederick, MD). Each testis (without mesonephros) was outlined twice, and the areas of these outlines were averaged to obtain an accurate measurement. The averages for the control testis organ cultures were set to 100%, and the area of each treated testis was calculated as a percentage of its paired control, as described elsewhere [40].

Whole-Mount Immunohistochemistry of Organ Cultures

After imaging, the organs from the VEGFR-TKI experiment were fixed overnight at 4°C in 4% paraformaldehyde. Samples were washed in PBS for 2 h at room temperature (RT), placed in blocking buffer (5% BSA, 0.1% Triton X-100, and PBS), and incubated overnight at 4°C, followed by 1 h at RT. Samples were then incubated overnight at 4°C with anti-PECAM1 antibody (Pharmingen, San Diego, CA; 1:50 dilution in blocking buffer), then rinsed with washing buffer (1% BSA, 0.1% Tween 20, and PBS) for 4-5 h at 4°C (changing the buffer once), followed by two 1-h rinses at RT. Samples were incubated overnight at 4°C with Cy5-conjugated secondary antibody (Jackson Laboratories, West Grove, PA; 1:500 in blocking buffer), then rinsed with washing buffer as described above. The above blocking, incubation, and washing steps were repeated using an anti-laminin primary antibody (DAKO, Carpinteris, CA; 1:100) and a Cy2-conjugated secondary antibody. Primary antibodies to PECAM1 (Pharmingen, San Diego, CA) and laminin (DAKO, Carpinteris, CA) were selected because they stain endothelial cells and basement membrane, respectively [41,42], allowing for visualization of vasculature and seminiferous cords.

The whole-mount immunohistochemistry procedure for the Je-11-treated organs was slightly modified to use Alexa Fluor secondary antibodies, in accordance with previously reported methods [43]. Organs were fixed in 4% paraformaldehyde for 1 h at RT [44], followed by two 5-min rinses in washing buffer (WB-PT) (1× PBS with 0.1% Triton X-100; Sigma, St. Louis, MO) and an additional 1-h rinse at RT. Tissues were then blocked in blocking buffer 1 (BB1) (WB-PT with 5% BSA; Invitrogen, Carlsbad, CA) for 1 h. Primary antibodies were diluted 1:50 in BB1 and incubated with gonads overnight at 4°C. On the second morning, organs were rinsed three times in blocking buffer 2 (BB2) (WB-PT with 1% BSA) for 30 min at RT before incubation with secondary antibodies Alexa Fluor 488 and Alexa Fluor 647 (1:200 in BB1; Molecular Probes, Eugene, OR) for 3-4 h at RT. Samples were then washed again in BB2 four times (30 min per wash), followed by a final rinse with 1× PBS.

All whole-mount immunohistochemistry samples were mounted in Gel/Mount (Biomeda, Foster City, CA) for subsequent confocal microscopy.

Confocal Microscopy of Organ Cultures

Images from the VEGFR-TKI whole-mount immunohistochemistry experiments were collected using a BioRad MRC1024ES confocal laser scanning microscope (Hercules, CA). Organs were scanned through a series of Z-sections from their dorsal to ventral surfaces at magnifications of 100×, 200×, and 600× (thickness of sections, 10, 5, and 3 μm, respectively) with green, red, and merged channels, respectively, to determine staining for PECAM and laminin. Organ depths were estimated at a magnification of 100× by the number of 10-μm Z-steps required to scan through the entire organ.

Images from the Je-11 and PI3K inhibitor (LY 294002) whole-mount immunohistochemistry experiments were collected using an Olympus FluoView 500 confocal laser scanning microscope. Organs were imaged and analyzed at magnifications of 100×, 200×, and 400× for morphological structures. We scanned throughout the depth and length of each organ to ensure that images were representative of gonadal morphogenesis. Organs were scanned from their dorsal to ventral surfaces at a magnification of 100× to determine organ depth. Depths were calculated for treated and control organs by the number of 3-μM sections required to scan through the entire organ. Images were taken with green, red, and merged channels open to determine staining for laminin, PECAM, and merged images, respectively.

Vascular Density Quantification

Red channel confocal images at 600× magnification were used to analyze the vascular density of control and VEGFR-TKI treated organ cultures and 400× magnification for Je-11-treated and P13K inhibitor organ cultures. Staining index (SI) was quantified with the use of Scion imaging. Densitometry was performed on 1-4 fields for each organ. Within each field, the SI was defined as the number of pixels exceeding an arbitrary grayscale value. The average SI for each organ was defined as the mean of the SIs for the fields measured. The mean vascular density value (i.e., SI) for each control testis organ culture was set to 100%, and the mean of each treated testis was calculated as a percentage of its paired control. Approximately 14 testis pairs were imaged for vascular density quantification for VEGFR-TKI, Je-11, and P13K inhibitor experiments.

Statistical Analysis

The mean value for each parameter evaluated in the control testis was set to 100%, and the mean of each treated testis was calculated as a percentage of its paired control. An analysis of variance was conducted to detect any statistical differences between means of the treated and control testes during analysis of vascular density, testis area, and organ depth, using JMP software (SAS Institute, Cary, NC). Dunnett test was then used to compare the percentage of

control to treated organs. Differences in data were considered to be statistically significant at a *P* value of <0.05, unless otherwise stated.

RESULTS

Pattern of Expression of mRNA for *Vegfa* Isoforms, *Kdr*, and *Flt1* During Testis Development

Vegfa188, *Vegfa164*, and *Vegfa120* mRNA were detected with conventional RT-PCR at E13 (Figure 1A). These three mRNA isoforms continued to be present until E18 during embryonic testis development. Five isoforms for *Vegfa* mRNA (*Vegfa205*, *Vegfa188*, *Vegfa164*, *Vegfa144*, and *Vegfa120*) were detected at E18 and continued to be expressed at P3 (Figure 1A). Thus, *Vegfa188*, *Vegfa164*, and *Vegfa120* mRNA were present around the time of seminiferous cord formation and may be involved in processes regulating the development of these morphological structures.

Conventional RT-PCR was conducted from E14 through E18 in the rat to determine whether receptors for VEGFA were present during embryonic testis development. *Kdr* mRNA was detected at all time points from E14 through E18 (Figure 1B). However, *Flt1* mRNA expression was not detected until E18. Therefore, the predominant action of VEGFA during embryonic testis development (neovascularization and morphogenesis [i.e., cord formation]) occurs through KDR (Figure 1B).

Localization of VEGFA and KDR

Immunohistochemistry was conducted from E14 through Postnatal Day (P) 5 to determine cell types expressing VEGFA and KDR during early testis morphogenesis. VEGFA expression was detected at E14 mainly in Sertoli cells (Figure 2, A and B), with some expression in germ cell cytoplasm within the seminiferous cords and in specific interstitial cells within the interstitium. Because the majority of the staining was detected within the nonvascularizing seminiferous cords, VEGFA may be involved in Sertoli-germ cell interactions that are independent from the usual angiogenic actions of VEGFA.

VEGFA staining was still present in the seminiferous cords at E16 (Figure 2, C and D), with more intense staining in Sertoli cells surrounding germ cells. Additional staining was detected within cells of the interstitium. By E19, VEGFA staining was still present in the cords, with less intense staining in the Sertoli cells and more intense staining in germ cell cytoplasm (Figure 2E). Certain interstitial cells also stained for VEGFA. In P0 testis, the pattern of VEGFA staining was similar to that in E19 testis (Figure 2F), except only specific germ cells had elevated levels of VEGFA staining in the cytoplasm at this age. By P5 (Figure 2G), Sertoli cells had markedly increased staining intensity, compared with earlier ages. VEGFA remained localized around specific germ cells. No staining was detected in the negative control E16 testis that was processed without primary antibody (Figure 2H).

KDR staining was present in seminiferous cords with some staining in interstitial cells (Figure 3, A and B) at E14. Interstitial cells, as well as Sertoli cells within seminiferous cords in E19 tissues (Figure 3C), were clearly expressing KDR protein. Interstitial cells, Sertoli cells, and cytoplasm of germ cells expressed KDR protein at P0 and P5 (Figure 3, D and E), whereas no expression was detected in E16 control testis sections that were processed without primary antibody (Figure 3F).

Effects of VEGFA Signal Inhibitors on Testis Morphogenesis in Organ Cultures

Treatment of E13 testis organ cultures with a VEGFA receptor signal transduction inhibitor, VEGFR-TKI (8 μM; Figure 4), severely perturbed (in 2 treated organs; Figure 4D) or totally inhibited (in 12 treated organs; Figure 4B) seminiferous cord formation, compared with the

findings for controls (Figure 4, A and C). There was no effect of VEGFR-TKI on testis morphogenesis at the 4-μM dose (data not shown). To further confirm KDR involvement, SU1498 (a specific signal transduction inhibitor for KDR) was administered at two doses (20 and 40 μM). There were no identifiable effects of SU1498 at 20 μM (data not shown); however, at 40 μM, SU1498 inhibited or severely perturbed seminiferous cord formation (Figure 4F), compared with the response in controls (Figure 4E).

To characterize the effects of the VEGFA signal transduction inhibitor, VEGFR-TKI, the area of testis organ cultures were measured, and treated organs were found to have a 23% reduction in size ($P < 0.01$; 100% vs. 77% ± 10%), compared with their paired controls. Furthermore, depth of organ cultures (detected by number of Z series confocal microscopy images conducted on each organ) was also reduced (by 21%) in the VEGFR-TKI-treated testis organ cultures ($P < 0.01$; 79% vs. 100% ± 2.3%), compared with their paired controls. Thus, it appears that disruption of VEGFA receptor signal transduction impairs the ability of seminiferous cords to form and reduces the area of the gonad.

In addition to evaluating the presence or absence of seminiferous cords, an objective of the current experiment was to determine the effects on vascular development in relation to seminiferous cord formation. After organ cultures were imaged for brightfield analysis, whole-mount immunohistochemistry was conducted for laminin (to determine the presence of seminiferous cords) and for PECAM (to determine alterations and patterns in vascular development). Confocal microscopy analysis demonstrated that control organ cultures had laminin staining (green; Figure 5, A-C), which outlined seminiferous cords, and PECAM-stained vascular tissue (red), which surrounded the seminiferous cords. Furthermore, at higher magnifications, mitotic germ cells within seminiferous cords also stained for PECAM (Figure 5C). In contrast, the treated organ was smaller, and there were no organized seminiferous cords (depicted by green basement membrane staining) or patterns of vascular development when viewed at higher magnifications (Figure 5, D-F). Also, because mitotic germ cells stain for PECAM, it was inconclusive whether endothelial cells were even present within the treated testis organ culture.

To quantify vascular density within control and VEGFR-TKI-treated organs, red channel confocal images at 600× magnification were evaluated in conjunction with Scion imaging. Three areas within all 14 testis pairs were imaged, and there was a 90% reduction in vascular density in VEGFR-TKI-treated organs, compared with paired control organs ($P < 0.01$; 10% vs. 100% ± 0.7%).

Effect of VEGFA Inhibitor, Je-11, on Testis Morphogenesis in Organ Cultures

To determine whether blocking VEGFA binding to its receptor would have similar effects as the VEGFA receptor signal transduction inhibitors, we used a VEGFA inhibitor, Je-11, which binds VEGFA and prevents it from binding to its receptor in testis organ cultures. At a 10-μM dose, Je-11 perturbed cord formation, resulting in abnormal or no cord formation (Figure 4, H and I), compared with controls (Figure 4G). Testis area ($P = 0.63$; $n = 14$ pairs) and depth ($P = 0.28$) were unaffected by treatment.

Normal vascular patterns, as determined by the presence of PECAM staining [45], were present in control organs surrounding seminiferous cords (Figure 5, G-I) and in the coelomic vessel (Figure 5, J-L). However, vasculature in treated organs was less dense, consisting of smaller microvessels (Figure 5, M-O). Vascular density, as indicated by PECAM expression, was reduced 42% in Je-11-treated organs ($P < 0.01$; 100% vs. 58% ± 10%; $n = 13$ organ pairs). Mitotic germ cells, as indicated by PECAM expression, were present in both treated and control testes (Figure 5, G-O). Therefore, differences in vascular density (PECAM staining) were attributed to modifications in vascular development.

Inhibition of PI3K Prevents Vasculogenesis and Cord Formation

The PI3K inhibitor LY 294002 was used to investigate VEGFA signal transduction pathways and whether the PI3K pathway was important for neovascularization and cord formation during testis development. E13 rat testes (n = 13 pairs for each dose) were either treated with 10 or 15 μM of LY 294002 or served as vehicle controls for a 3-day culture and were analyzed for cord formation, testis depth, area, and PECAM staining density. Seminiferous cords developed normally in all controls. No differences in testis depth (P = 0.21; n = 13), area (P = 0.086; n = 13), or overall vascular (PECAM) density (P = 0.17; n = 13) resulted from treatment with 10 μM of LY 294002. However, cords in treated organs appeared smaller and irregular in shape in response to the inhibitor at both doses (Figure 6, B and D), compared with the corresponding controls (Figure 6, A and C). Testis depth was not affected by 15 μM of LY 294002 treatment (P = 0.62; n = 15 pairs), but testes area was reduced by 23.8% (P < 0.01; n = 14 pairs), compared with vehicle controls.

Analysis of PECAM expression revealed the presence of organized mitotic germ cells and vasculature throughout treated and control testes (Figure 7, A-F). Distinct vascular patterns were organized around seminiferous cords in control testes (Figure 7, B and C) but were lacking in treated testes (Figure 7, E and F). Scion imaging revealed higher vascular (PECAM) density in control organs than in those treated with 15 μM of LY 294002. In fact, vascular (PECAM) density was reduced by 46%, compared with that in controls (P < 0.01; n = 15) (Figure 7). At the 15-μM dose, the LY 294002 PI3K inhibitor affected normal testis cord morphologic characteristics and reduced vascular density. Thus, blocking the PI3K signal transduction pathway arrests both cord formation and vascular density.

DISCUSSION

Although the role of many growth factors have been investigated and implicated in testis morphogenesis, data from the current study support that VEGFA and KDR are involved in both neovascularization and seminiferous cord formation in the embryonic testis. Potentially, these effects are mitigated through the PI3 kinase pathway. These experiments are also the first to support the notion that vascular development and seminiferous cord formation may be dependent events during testis development. Furthermore, we also provide evidence supporting the concept that VEGFA, a Sertoli-cell secreted factor, potentially directs mesonephric cell migration, resulting in the formation of seminiferous cords and development of sex-specific patterns of vasculature.

Vascular development within the testis does not appear to be dependent on *Sry*, because male patterns of vascular development can form in XX *Wnt4* knockout mice (*Wnt4*$^{-/-}$) [46]. Also, overexpression of *Wnt4* in XY gonads disrupts vascular development [47]. It is proposed that expression of *Sry* antagonizes *Wnt4* and the gene encoding follistatin (downstream from *Wnt4*) to allow for endothelial cell migration and formation of male-specific vascular development during testis morphogenesis. In the current study, we determined that several *Vegfa* gene isoforms are expressed in the indifferent gonad. We propose that regulation of expression of VEGFA and its receptors, KDR and FLT1, may be modified by genes differentially expressed in the XX versus XY gonad to elicit sex-specific vascular patterns.

Hypoxia [48-50], estradiol [51,52], progesterone [53], and factors signaling through the BMP/SMAD pathway [54,55] have all been implicated in increased or decreased expression of VEGFA. The *Vegfa* gene contains eight exons, which, through alternative splicing, can produce many VEGFA isoform proteins that have angiogenic and antiangiogenic properties. In the current study, the three mRNA isoforms *Vegfa120*, *Vegfa164*, and *Vegfa188* were expressed during seminiferous cord formation. These three isoforms have different diffusion affinities, because of the absence or presence of one or multiple heparin binding domains. Thus, they

potentially work together, presumably in the absence of *Wnt4* expression, as a chemoattractant gradient to induce and recruit mesonephric endothelial cell migration into the developing testis establishing male-specific vascular development [56-59]. VEGFA appears to work through KDR to cause neovascularization of the testis. In the current study, mRNA for *Flt1* was not expressed until E16 of testis development, well after seminiferous cord formation, whereas *Kdr* was present throughout all embryonic time points evaluated. Similarly, in other tissues and organs, KDR is the primary receptor that facilitates VEGFA-induced neovascularization [60].

In addition to *Vegfa120*, *Vegfa164*, and *Vegfa188*, other *Vegfa* mRNA isoforms (*Vegfa144* and *Vegfa205*) appeared by E18 within the embryonic testis. The presence of these other isoform bands suggests that alternative splicing occurs after E18 to produce a greater number of *Vegfa* isoforms. Several events occur at this time during rat testis development. Steroidogenic enzymes, such as hydroxysteroid dehydrogenase 3-β and aromatase, appear in differentiating Leydig cells within the rat during E15-16 [61]. The *Vegfa* promoter region has been demonstrated to have several nonconcensus ERE binding sites, and both estrogen and progesterone treatment in breast cancer and uterine cells has altered expression of *Vegfa* mRNA isoforms [52]. Therefore, steroid hormones expressed at this time during testis development may alter expression of specific *Vegfa* isoforms.

A second possibility is that VEGFA may be involved in germ-Sertoli cell interactions leading to maturation of germ cells. At E16.5 in the mouse (which translates to E17-18 in the rat), gonocytes (specific stage germ cells) undergo mitotic arrest in the G0/G1 phase of the cell cycle [62]. Other investigators have determined that VEGFA may be important in germ cell survival and development, because expression of VEGFA receptors are localized to specific stages of germ cells in late gestation and early postnatal development [63]. VEGFA [63], prokineticin 1 (also known as EG-VEGF), and prokineticin 2 (formerly known as Bv8) [64-66] have all been demonstrated to be involved in germ-Sertoli cell interactions in the postnatal testis. Therefore, it is possible that, after sex-specific vasculature has formed, VEGFA is primarily involved in germ cell maturation/survival mechanisms occurring during spermatogenesis.

In the current study, VEGFA protein expression was localized to the testicular cords and, specifically, the Sertoli cells early in testis development. This finding is significant, because the Sertoli cell is the first cell to differentiate in the testis and has been proposed to regulate genes controlling testis differentiation [4,5]. Endothelial and pre-endothelial cells express KDR [60]. Thus, one purpose of Sertoli cell secretion of VEGFA may be to induce endothelial cells or endothelial cell precursors to migrate into the differentiating testis to aid in neovascularization and cord formation. Other growth factors, such as PDGFA, have been demonstrated to be important for male specific cell migration [23]. Although VEGFA is thought to be critical to endothelial cell migration [26,67], PDGFA is thought to cause migration of pericytes that form a smooth muscle layer around blood vessels to support and maintain the integrity of vasculature [68-70]. It is possible that pre-endothelial cells are migrating toward a concentration gradient of VEGF and that, as these cells migrate, they differentiate into endothelial and preperitubular cell types that allow for seminiferous cord formation and sex-specific vasculature to develop.

Another purpose of VEGFA during cord formation may be to allow for differentiation of endothelial cells into arterial networks. Before cord formation in the mouse (on E11.5), pre-endothelial cells express markers for both venous (EPHB4) and arterial (ephrin B2) markers. However, just after E11.5, a rapid reorganization of vasculature occurs within the testis to form arterial networks, which surround the developing seminiferous cords and coelomic blood vessel [71]. Thus, after cord formation, the testis is mainly composed of arterial networks

instead of veins. Several scientists have established that VEGFA is the main growth factor involved in differentiating endothelial cells into arterial networks [72,73]. Therefore, in addition to establishing a vascular network, VEGFA may also act to induce endothelial cells to differentiate into arteries forming the arterial network present after cord formation.

Inhibition of VEGFA's action through a VEGFA receptor signal transduction inhibitor, VEGFR-TKI (which blocks the actions of both FLT1 and KDR), inhibited seminiferous cord formation in testis organ cultures and inhibited sex-specific vascular development. Furthermore, a KDR-specific inhibitor arrested cord formation. The fact that both inhibitors yielded similar results was expected, because the only receptor detected during cord formation was KDR. To further support VEGFA's role in testis morphogenesis, a VEGFA antagonist, Je-11, also impaired normal cord formation and reduced vascular density, as measured by PECAM staining. Je-11 acts to inhibit VEGFA from binding to its receptors. Therefore, sex-specific vascular development, vascular density, and seminiferous cord formation were inhibited when VEGFA signal transduction or binding to its receptor was prevented.

The PI3K signal transduction pathway has been implicated in many different systems to be important in VEGFA mediated endothelial cell survival, proliferation, and migration [74-77]. Previous research has demonstrated that the PI3K specific inhibitor, LY 294002, inhibits seminiferous cord formation in rat testis cultures. Cell migration assays revealed that testis morphogenesis impaired by LY 294002 was due to inhibition of mesonephric cell migration [78]. Because inhibition of VEGFA perturbed cord formation and vascular development, we sought to determine whether inhibition of the PI3K signal transduction pathway would also affect vascular development in addition to seminiferous cord formation. PI3K inhibition reduced vascular density and testis area and perturbed cord formation in a manner that was similar to our results of VEGFA signal transduction and antagonist inhibition. Thus, we believe that these studies provide correlative data to demonstrate that VEGFA may work predominately through the PI3K pathway to elicit its effects during testis morphogenesis. Furthermore, because both cord formation and vascular development is arrested by inhibition of PI3K, this further links these two morphogenic events through a single signal transduction pathway.

VEGFA is not the only growth factor that elicits its effects through the PI3K pathway. Neurotrophin 3 [21,78] and PDGFA [78-80] have all been implicated to act through PI3K to elicit their effects. Thus, multiple growth factors may work through PI3K in order to contribute to regulation of testis morphogenesis.

In conclusion, the current studies demonstrate that VEGFA and its receptor KDR are expressed at the time of testis morphogenesis (i.e., seminiferous cord formation and sex-specific vascular development). VEGFA is expressed by Sertoli cells and other cells within the seminiferous cords early in development, with expression of KDR occurring in the cords and in cells that we suspect to be the developing vasculature. The importance of VEGFA in testis formation was underscored by the disruption of both seminiferous cord formation and vascular development in testis organ cultures following treatment with specific VEGFA inhibitors and VEGFA receptor signal transduction inhibitors. These studies establish a role for VEGFA in vascular development during testis morphogenesis and also support coordinate regulation of cord formation and sex-specific vascularization through the PI3 kinase pathway.

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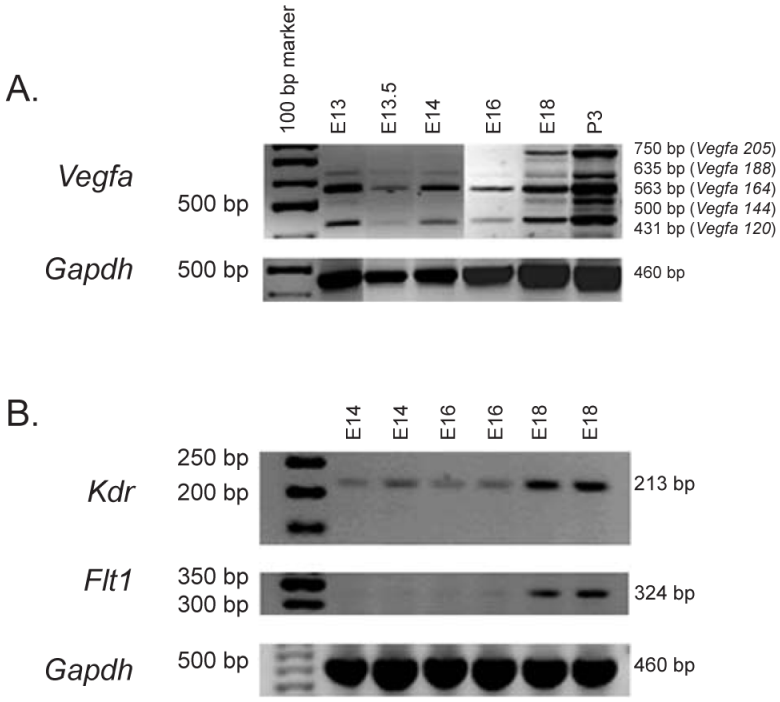

Figure 1.

Conventional RT-PCR for VEGFA isoforms from Embryonic Day (E) 13 to Postnatal Day 3 of testis development (**A**) and *Kdr* and *Flt1* from E14 through E18 of testis development (**B**). Negative controls (not shown) had no template and did not result in a band. RT-PCR was conducted on three to five different samples for each time point during testis development.

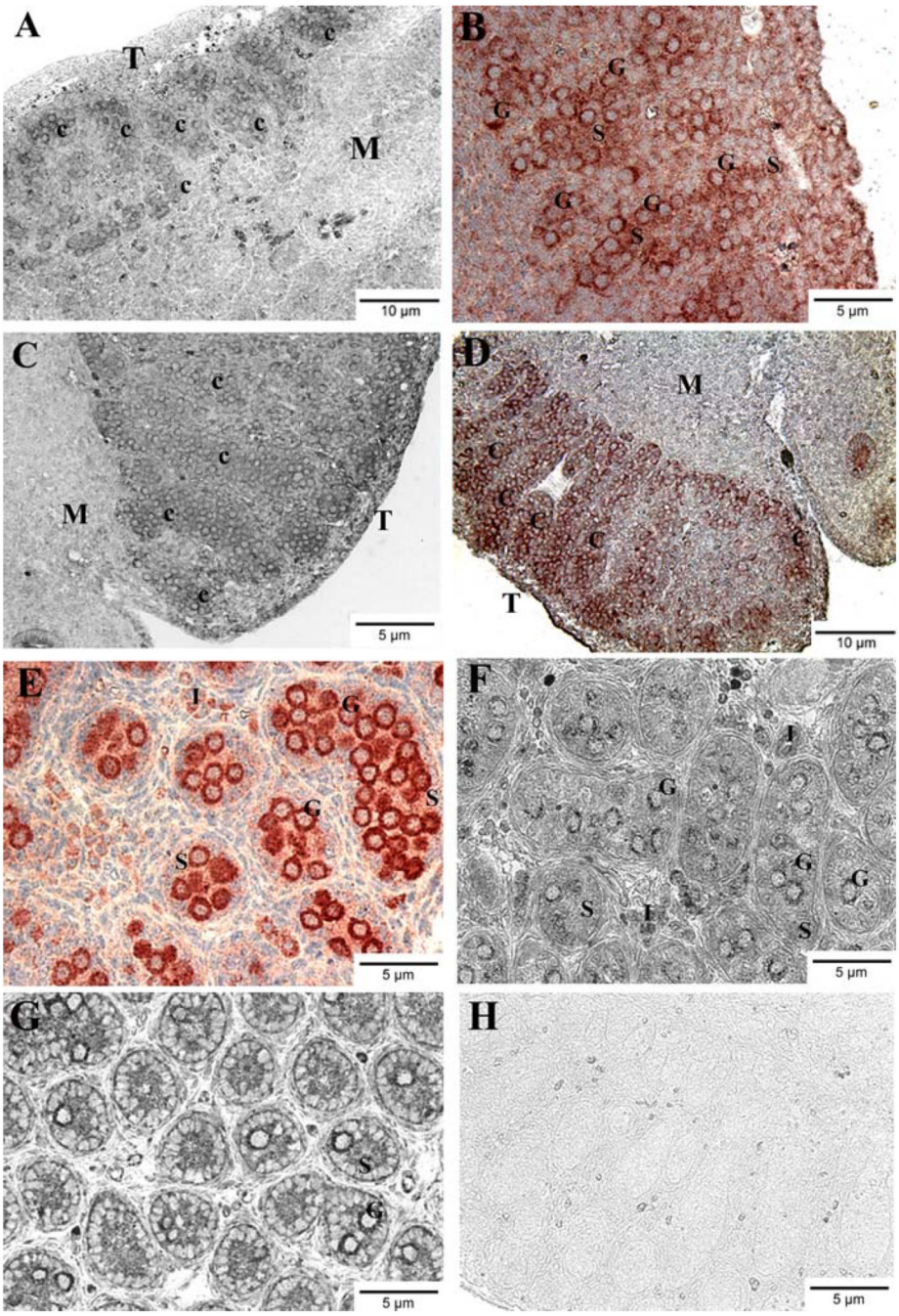

Figure 2.

Immunohistochemistry for VEGFA in Embryonic Day (E) 14 testis (magnification, ×200) (**A**), E14 (×400) (**B**), E16 (×400) (**C**), E16 (×200) (**D**), E19 (×400) (**E**), P0 (×400) (**F**), and Postnatal Day 5 (×400) (**G**). E16 sections with no primary antibody (**F**) served as negative controls. These results are representative images from 3 different experiments conducted with three to six different sections of tissue from each age group. T = testis; M = mesonephros; c = cord; i = interstitium; S = Sertoli; G = germ.

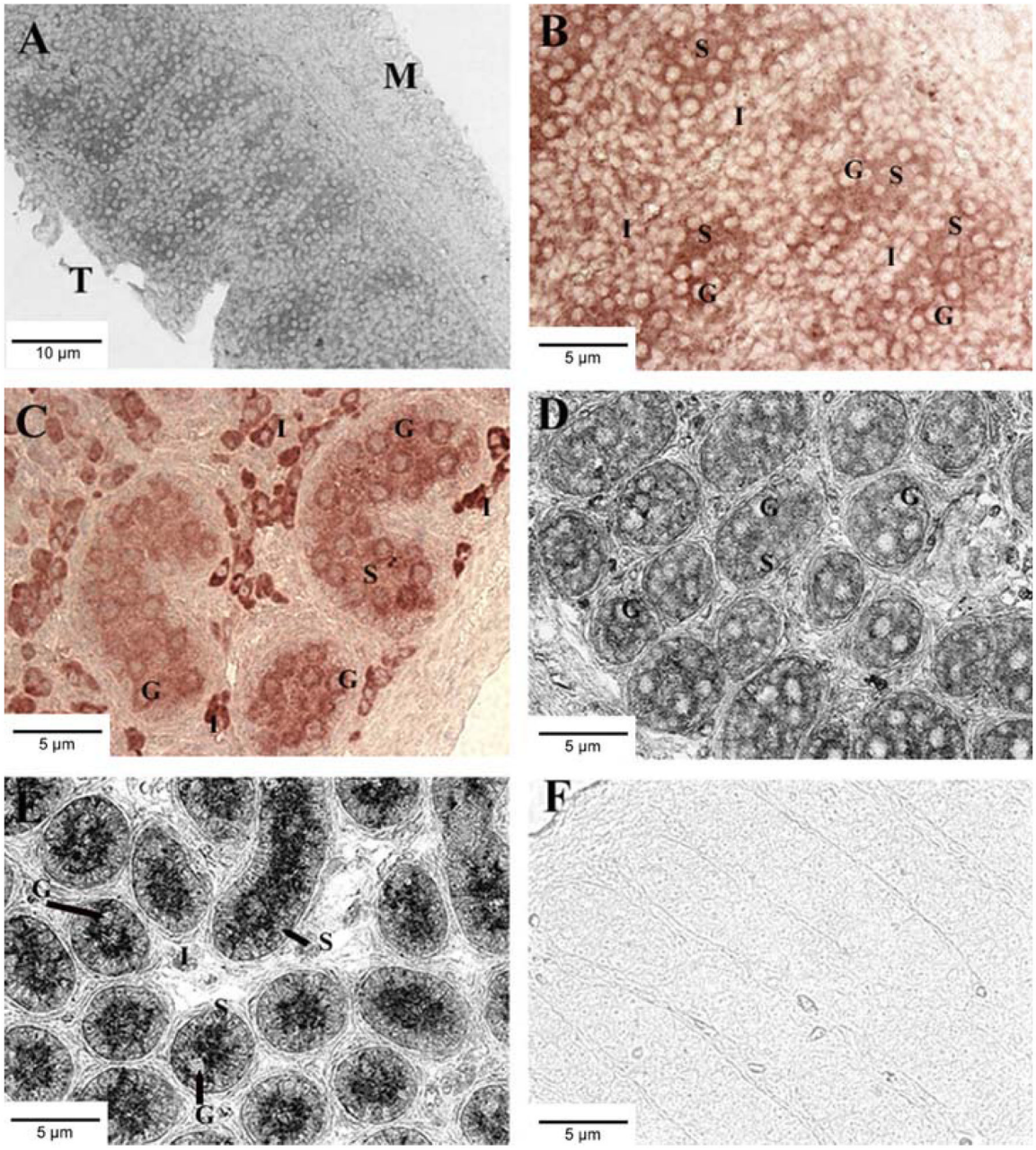

Figure 3.

Immunohistochemistry for KDR (VEGFR2) expression in testes at Embryonic Day (E) 14 (magnification, ×200) (**A**), E14 (×400) (**B**), E19 (×400) (**C**), Postnatal Day (P) 0 (×400) (**D**), and P5 (×400) (**E**). E16 controls with no primary antibody (**F**) served as negative controls. These results are representative images from three different experiments conducted with three to six different sections of testis tissue from each age group. T = testis; M = mesonephros; c = cord; i = interstitium; S = Sertoli; G = germ. Arrows point to Sertoli and germ cells.

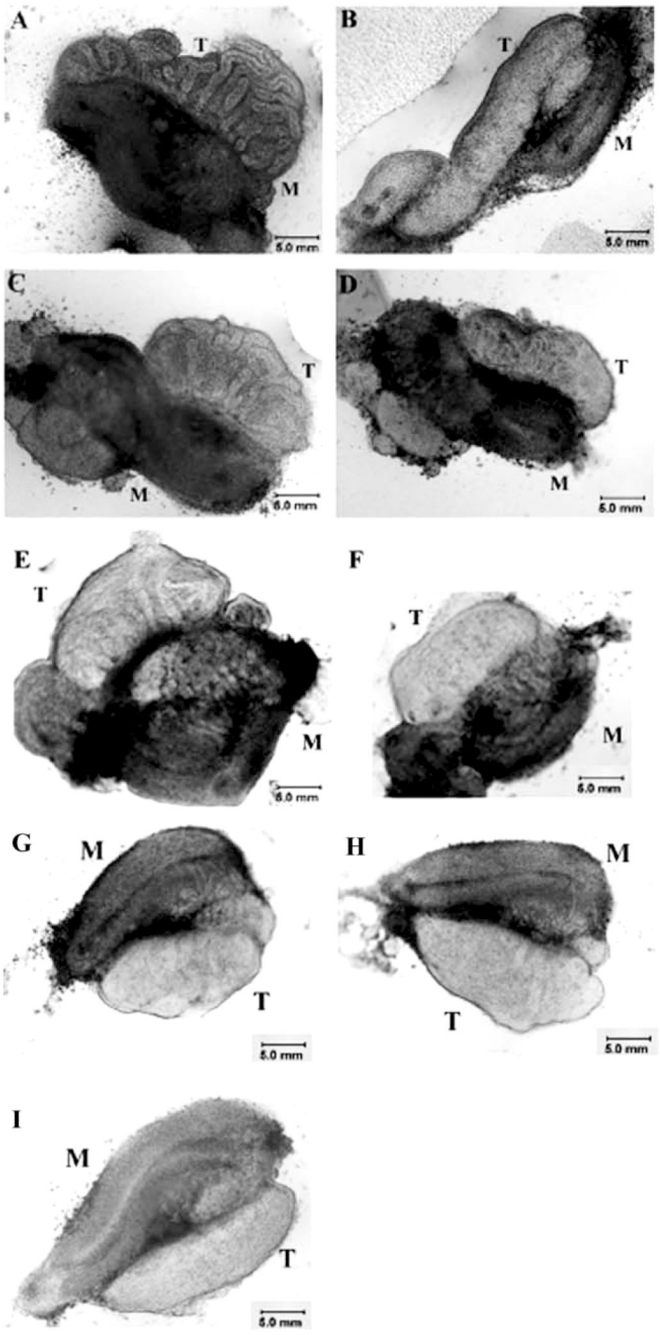

Figure 4.
Effect of the VEGFR signal transduction inhibitors VEGFR-TKI, SU1498, and Je-11 on seminiferous cord formation in organ cultures of rat testis obtained on Embryonic Day 13. Organ cultures contained vehicle controls (DMSO) (**A, C, E, G**) or specimens treated daily with 8 μM of VEGFR-TKI (**B, D**), 40 μM of SU1498 (**F**), or 10 μM of Je-11 (VEGFA antagonist) (**H, I**). T = testis; M = mesonephros.

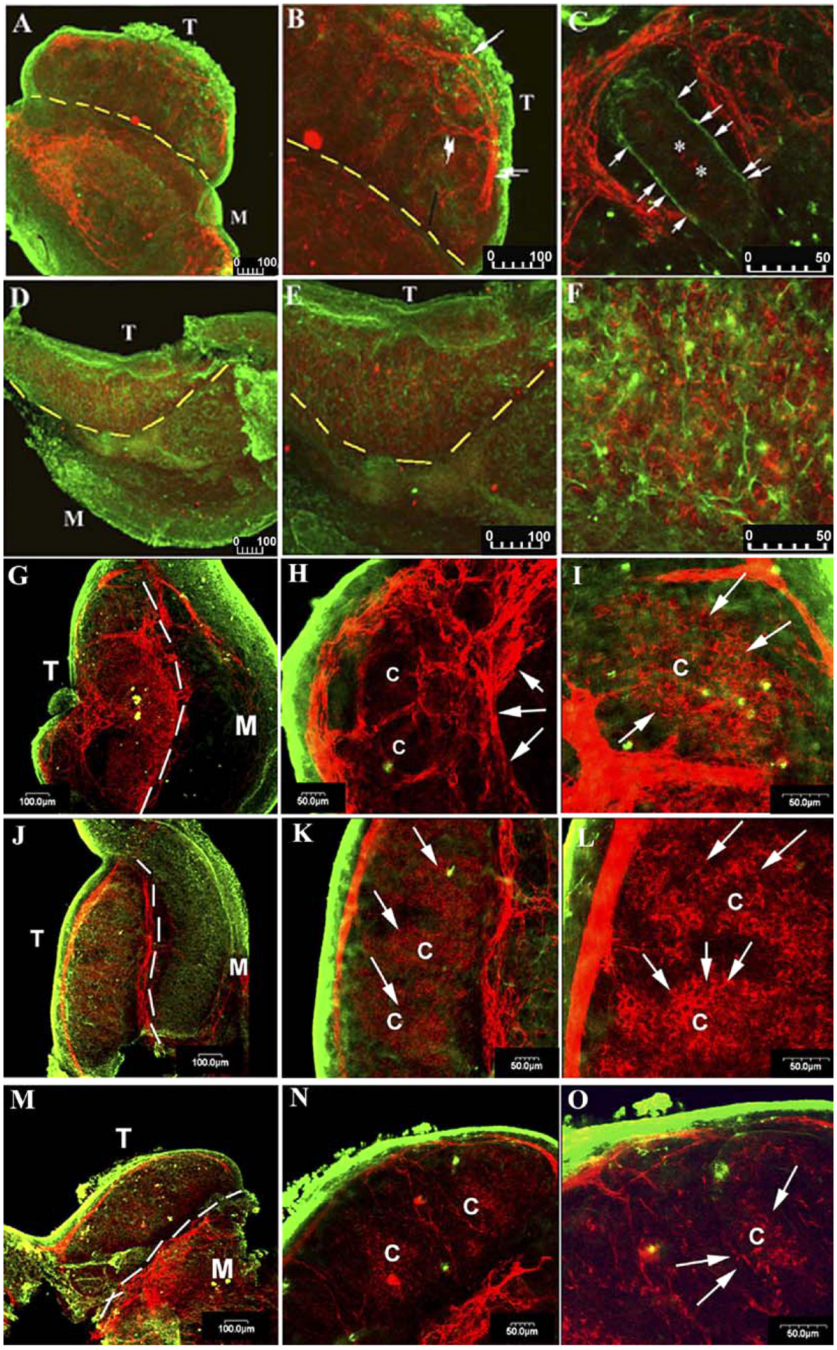

Figure 5.

Whole-mount immunohistochemistry of Embryonic Day 13 testis organ culture controls for VEGFR-TKI treatments (**A**, **B**, **C**), testis cultures treated with 8 μM VEGFR-TKI (**D-G**), vehicle controls for VEGFA antagonist Je-11 (**G-L**), and testis cultures treated with 10 μM Je-11 (**M-O**). Cultured organs were labeled with antibodies against laminin (green, basement membrane marker) and PECAM (red, endothelial cell and mitotic germ cell marker). Note that only mitotic germ cells express PECAM; germ cells that are not mitotic do not express PECAM. A representative control organ (**A-C**), shows both blood vessel patterns (arrows, **B**) and seminiferous cord formation (arrows, **C**). Mitotic germ cells can be seen within the

seminiferous cords (*, **C**). Organs treated with 8 μM VEGFR-TKI (**D-F**) do not have normal vasculature patterns or cord formation.

Magnifications are ×100 (**A, D**), ×200 (**B, E**), and ×600 (**C, F, G**). Normal vascular patterns surrounding seminiferous cords within the testis are detected in vehicle controls for Je-11 at magnifications of ×100 (**G, J**), ×200 (**H, K**), and ×400 (**I, L**). At high magnifications (**I, K, L**), mitotic germ cells (arrows) have organized into cord-like structures and are surrounded by vasculature. The coelomic vessel also expressed PECAM (K, L). In Je-11-treated testes, germ cells are clustered together into smaller, irregular cord patterns with reduced vascular density at magnifications of ×100 (**M**), ×200 (**N**), and ×400 (**O**). T = testis; M = mesonephros; c = cord; CV = coelomic vessel. Dashed lines indicate the boundary between the testis and mesonephros.

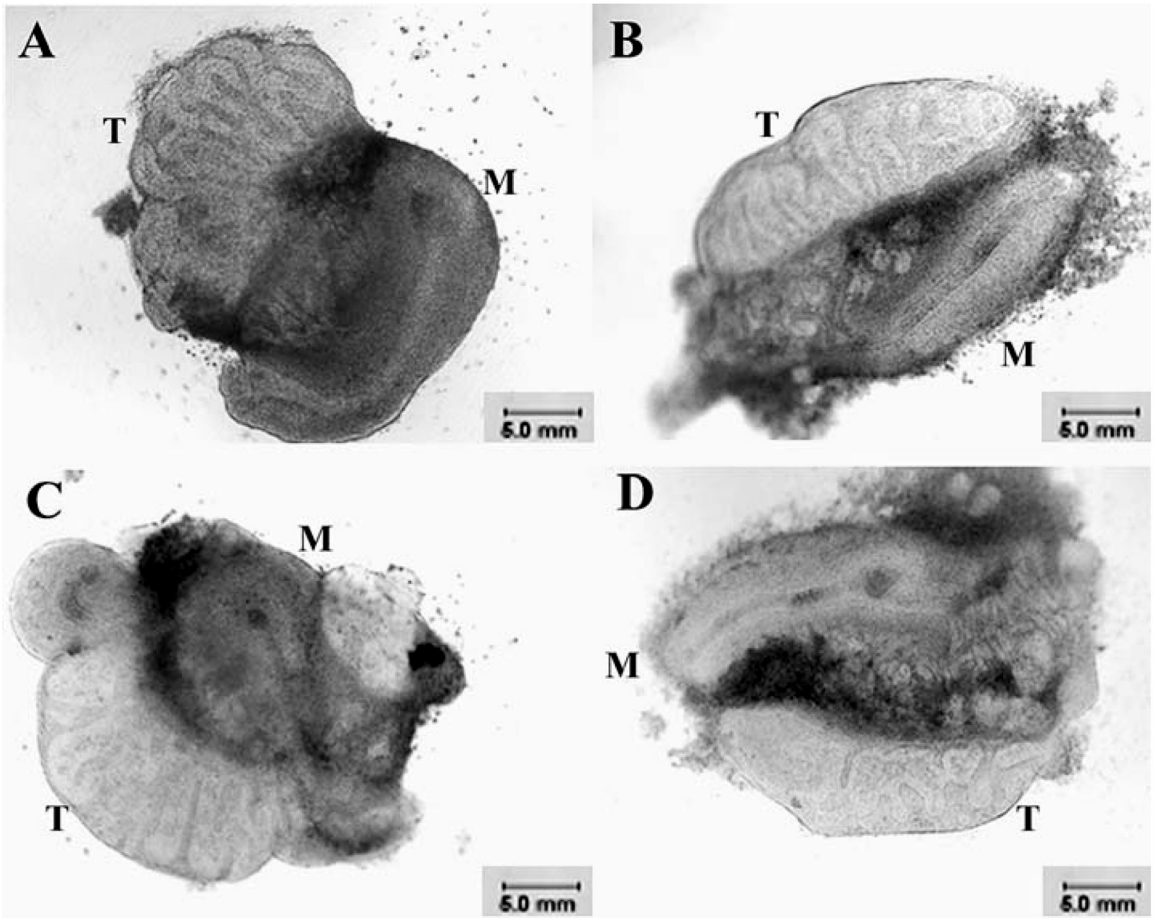

Figure 6.

Effects of LY 294002 on organ cultures rat testis obtained on Embryonic Day (E) 13. Cultured testes from E13 rats treated with DMSO vehicle control (**A**, **C**) developed normal seminiferous cords, whereas testes treated with 10 μM (**B**) or 15 μM (**D**) of the PI3K inhibitor LY 294002 developed smaller and occasionally swollen cords (**B**) or had perturbed cord formation (**D**). T = testis; M = mesonephros.

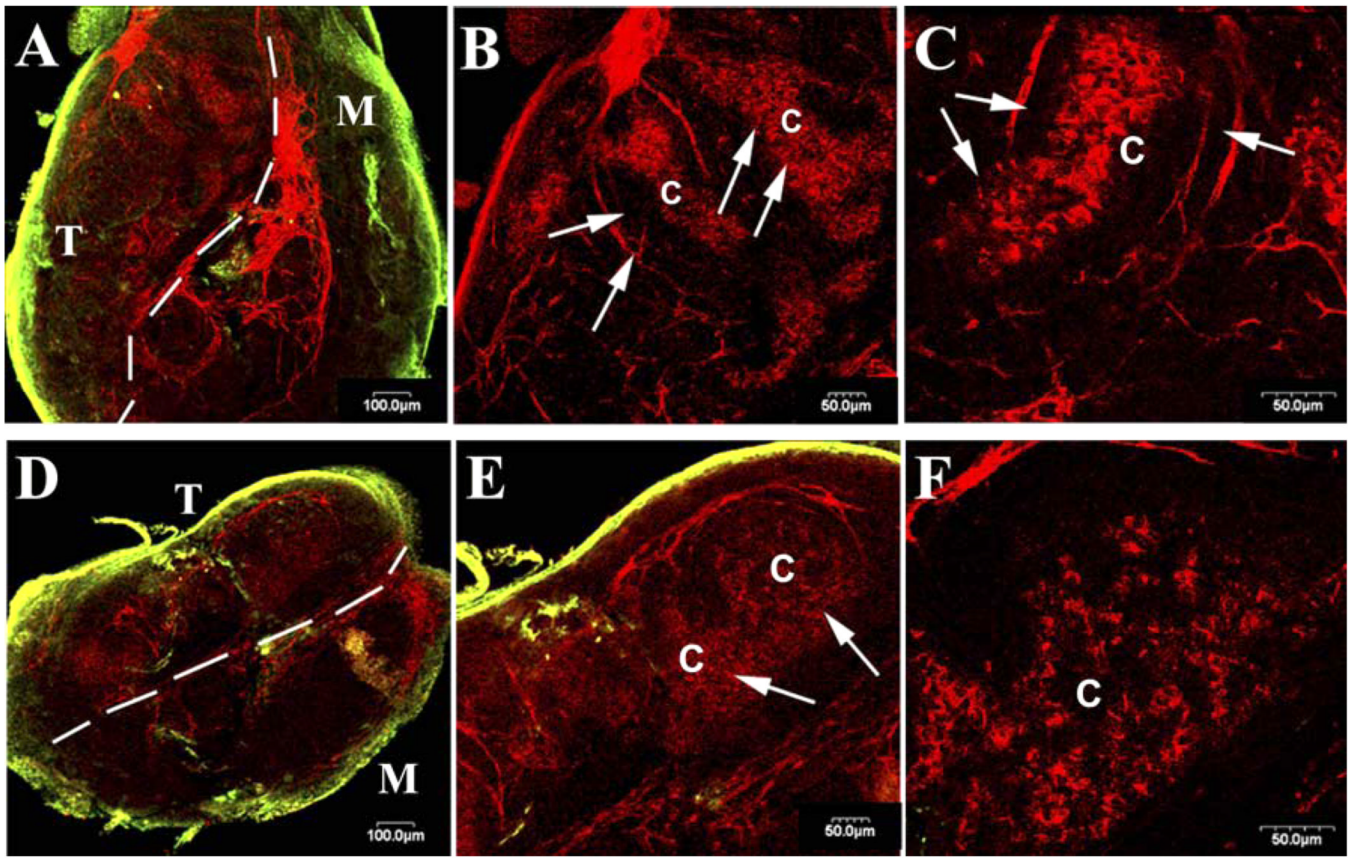

Figure 7.

Effect of 15 μM of LY 294002 on whole-mount immunohistochemistry on Embryonic Day 13 rat testis and mesonephros organ cultures. A representative control organ (**A**-**C**) with germ cells is organized within cords surrounded by vasculature (arrows). The treated testis (**D**-**F**) is smaller than the control, and germ cells are not as clearly organized within cords. Vasculature surrounds these cords (**F**) but is less abundant than that in paired control testes. T = testis; M = mesonephros; c = cords.