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Ningxia Lu
University of Nebraska-Lincoln

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

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

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

Vanessa M. Brauer
University of Nebraska-Lincoln

See next page for additional authors

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Loss of Vascular Endothelial Growth Factor A (VEGFA) Isoforms in the Testes of Male Mice Causes Subfertility, Reduces Sperm Numbers, and Alters Expression of Genes That Regulate Undifferentiated Spermatogonia

Ningxia Lu, Kevin M. Sargent, Debra T. Clopton, William E. Pohlmeier, Vanessa M. Brauer, Renee M. McFee, John S. Weber, Napoleone Ferrara, David W. Silversides, and Andrea S. Cupp

Department of Animal Science (N.L., K.M.S., D.T.C., W.E.P., V.M.B., R.M.M., A.S.C.), University of Nebraska-Lincoln, Lincoln, Nebraska 68583–0908; Winona State University (J.S.W.), Winona, Minnesota 55987; University of California-San Diego School of Medicine (N.F.), La Jolla, California 92093; and Department of Veterinary Biomedicine (D.W.S.), Faculty of Veterinary Medicine, University of Montreal St-Hyacinthe, Québec, Canada J2S 2M2

Corresponding author.
Address all correspondence and requests for reprints to: Andrea S. Cupp, Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE 68583–0908., E-mail: ude.lnu@2ppuca.
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Abstract

Vascular endothelial growth factor A (VEGFA) isoform treatment has been demonstrated to alter spermatogonial stem cell homeostasis. Therefore, we generated *pDmrt1-Cre;Vegfa*$^{-/-}$ (knockout, KO) mice by crossing *pDmrt1-Cre* mice to floxed *Vegfa* mice to test whether loss of all VEGFA isoforms in Sertoli and germ cells would impair spermatogenesis. When first mated, KO males took 14 days longer to get control females pregnant ($P < .02$) and tended to take longer for all subsequent parturition intervals (9 days; $P < .07$). Heterozygous males sired fewer pups per litter ($P < .03$) and after the first litter took 10 days longer ($P < .05$) to impregnate females, suggesting

a more progressive loss of fertility. Reproductive organs were collected from 6-month-old male mice. There were fewer sperm per tubule in the corpus epididymides ($P < .001$) and fewer ZBTB16-stained undifferentiated spermatogonia ($P < .003$) in the testes of KO males. Testicular mRNA abundance for *Bcl2* ($P < .02$), *Bcl2:Bax* ($P < .02$), *Neurog3* ($P < .007$), and *Ret* was greater ($P = .0005$), tended to be greater for *Sin3a* and tended to be reduced for total *Foxo1* ($P < .07$) in KO males. Immunofluorescence for CD31 and VE-Cadherin showed no differences in testis vasculature; however, CD31-positive staining was evident in undifferentiated spermatogonia only in KO testes. Therefore, loss of VEGFA isoforms in Sertoli and germ cells alters genes necessary for long-term maintenance of undifferentiated spermatogonia, ultimately reducing sperm numbers and resulting in subfertility.

Male-related factors such as low sperm count and abnormal spermatogenesis are responsible for 50% of the infertility afflicting 2.1 million U.S. couples. The incidence of male infertility cases has increased, resulting in the new disorder—testicular dysgenesis syndrome (1). Because testicular dysgenesis syndrome can involve a range of little to aberrant spermatogenesis (2), a misregulation of spermatogonial stem cell (SSC) homeostasis is a possible contributor. Little is known about factors involved in development of SSCs; however, we do know SSCs differentiate from primitive germ cells called gonocytes that must migrate from the center of the testicular cords (tubules) to the periphery during testis development and that they also undergo proliferation (self-renewal). This process occurs around postnatal days 3–5 in rodents after mitotic arrest that begins around embryonic day (E) 17 (3–5). If no gonocyte migration occurs, these cells undergo apoptosis and the population of SSCs does not form. Thus, factors that affect SSC formation may also affect expansion of SSCs, whereas inhibition of these factors may cause apoptosis and a reduced SSC pool. A balance between SSC self-renewal and differentiation is needed to maintain spermatogenesis and male fertility.

Vascular endothelial growth factor A (VEGFA) is a paracrine growth factor responsible for blood vessel development as well as endothelial cell migration. Although VEGFA isoforms are primarily thought of as regulatory factors for blood vessel growth and maintenance, their biological activities extend well beyond vascular biology. We have demonstrated that altering VEGFA isoform activity in the testis in vivo results in significant changes in the ability of SSCs to self-renew and colonize seminiferous tubules (6). Furthermore, expression of VEGFA in normal testes, prostate, seminal vesicles, and semen suggests that VEGFA may be involved in male fertility (7, 8).

The *Vegfa* gene is composed of eight exons that can be alternatively spliced to produce different proangiogenic and antiangiogenic isoforms. In general, proangiogenic isoforms promote development of vasculature, whereas antiangiogenic isoforms inhibit vascular development (9, 10). Studies have demonstrated VEGFA-positive staining surrounding specific germ cells and in some germ-cell cytoplasm at E14, E16, E19, postnatal day (P) 5 and 5 (11). Expression of VEGFA_164 was in germ cells from P3-P5 in mice at the time of gonocyte-to-SSC transition and was reduced thereafter. In addition, antiangiogenic VEGFA isoforms were in some germ cells at E14 and E16, the interstitium and faintly in Sertoli and germ cells at P0, and undifferentiated spermatogonia after P5 and in primary spermatocytes and round spermatids at P20 (6, 12). Recent data from our laboratory have demonstrated that VEGFA isoforms can affect SSC renewal and proliferation depicted through SSC transplants into recipients. The

antiangiogenic isoform, VEGFA_165B, inhibited SSC colonization, suggesting that they inhibit SSC self-renewal or stimulate SSC differentiation. Antibodies to the proangiogenic isoform, VEGFA_164, also inhibited formation of colonies in recipients, indicating that VEGFA_164 is necessary for SSC renewal (6). Taken together, these data suggest that a balance of VEGFA isoforms is necessary to regulate the SSC pool and male reproductive lifespan.

For the current study, we proposed that VEGFA plays an important role in SSC renewal and differentiation. To investigate the effects of VEGFA in vivo, we generated Sertoli-germ-cell-specific VEGFA isoform null (both proangiogenic and antiangiogenic) transgenic mice using a *pDmrt1-Cre*. The objective of the present study was to determine the in vivo effects of Sertoli-germ-cell-specific VEGFA loss on male reproductive phenotypes including genes involved in the regulation of undifferentiated spermatogonia, spermatogenesis, and testis morphogenesis.

Materials and Methods

Animals

Sertoli and germ-cell VEGFA knockout (KO) mice were obtained using the Cre-lox approach using a line of *Vegfa* floxed mice (13) mated with pig doublesex and mab-3-related transcription factor 1 (*Dmrt-1*-1) promoter (*pDmrt-1*)-Cre mice (14). Homozygous *Vegfa* floxed mice were mated to mice positive for the *pDmrt-1-Cre* allele. The resulting F1 heterozygous mice were first mated back to the homozygous *Vegfa* floxed founders to generate the F2 population and then were collected for the study (see Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Control and homozygous KO mice were from the F2 generation. We used Cre-negative $Vegfa^{-/-}$ or $Vegfa^{+/-}$ mice as controls and Cre-positive $Vegfa^{-/-}$ mice as KOs (*pDmrt1-Cre;*$Vegfa^{-/-}$) or Cre-positive $Vegfa^{+/-}$ as heterozygotes (Het). *Dmrt1* is expressed in the indifferent mouse gonad at E10.5 in precursor cells that differentiate into Sertoli cells in males and granulosa cells in females. Murine *Dmrt1* is expressed in Sertoli cells and in germ cells within testes and in somatic and germ cells within ovaries at E12.5. By E13.5, expression is no longer detectable in ovarian somatic cells. Expression of *Dmrt1* in the testis increases from E13.5 through E15.5. Germ cell expression of *Dmrt1* mRNA is detectable in premeiotic spermatogonia (15), and expression has been detected in Sertoli cells of all stage tubules up to the first spermatogenic wave (16, 17). The *pDmrt1* gene is expressed similarly to mouse *Dmrt1* (14). In our *pDmrt1-Cre;*$Vegfa^{-/-}$ mouse, Cre recombinase activity is driven by the *pDmrt1* promoter and excises exon 3 of the *Vegfa* gene. Exon 3 is present in both proangiogenic and antiangiogenic isoforms; thus, all *Vegfa* isoforms are affected in the cells where *Dmrt1* is expressed. Body and organ weights were evaluated in 6-month-old male mice (n = 9 controls, n = 8 KO), and at kill, testes, epididymides, prostates, seminal vesicles, kidneys, and adrenals were collected for analysis.

All animal protocols were approved by the University of Nebraska Institute of Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal models were procured from collaborating investigators with verbal or written MTA agreements. Thus, the corresponding author does not have rights to freely distribute these mouse lines.

Genotyping

Genomic DNA was extracted from tail samples by proteinase K digestion, followed by 6 M NaCl extraction and ethanol precipitation for genotyping via conventional PCR. DNA was amplified for 30 cycles after an annealing temperature of 54°C to detect floxed *Vegfa*. Expression of *pDmrt1-Cre* was detected from DNA annealed at 55°C and amplified for 34 cycles with a forward primer for *pDmrt1* and a reverse for *Cre*.

After RNA was extracted from testes and converted to cDNA, RT-PCR was also used to determine gonadal *Cre* expression in each of the collected organs. All males that did not have *Cre* expressed within testes or that had *Cre* expressed in other organs were removed from analysis. Sequences for the primers used in PCR can be found in Supplemental Table 1.

Hormone assays

Trunk blood was collected at the time of euthanasia and placed into tubes with 20 μL potassium EDTA solution (0.01 M EDTA diluted 30% in PBT; Fisher Scientific). Blood samples were immediately centrifuged at 1250*g* for 10 minutes. The separated plasma was then collected into a new tube and stored at −80°C. Testosterone concentrations were determined by a testosterone ELISA kit (catalog no. 1880; Alpha Diagnostics International Inc) (18). A standard curve was established using 0 ng/mL, 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL, 10 ng/mL, and 20 ng/mL samples. Assay sensitivity was 0.125 ng/mL and intra-assay coefficient was 4.29%.

Fixation, embedding, staining, and immunohistochemistry

Representative tissues were fixed in Bouin's solution and paraffin embedded according to standard procedures (19). Tissues were sectioned (5 μm), and immunohistochemistry (IHC) was performed as previously described (19). The VEGFA antibody was a rabbit polyclonal IgG raised against a peptide corresponding to amino acids 1–140 of human VEGFA (catalog no. sc-507; Santa Cruz Biotechnology). It served as a pan-antibody for both proangiogenic and antiangiogenic isoforms to determine if any VEGFA isoforms were present in Sertoli and germ cells within KO and control mouse testes. The primary antibody was diluted 1:100 in 10% normal goat serum (NGS). As a negative control, serial sections were processed without primary antibody. Biotinylated goat antirabbit secondary antibodies were diluted 1:300 in 10% NGS and used with each primary antibody in this study (catalog no. BA-1000; Vector Laboratories). The secondary antibody was detected using aminoethyl carbazole chromagen substrate solution (Invitrogen). Similar immunohistochemistry protocols were used for additional primary antibodies. The zinc finger and BTB domain containing 16 (ZBTB16, also known as PLZF) primary antibody (catalog no. sc-22839; Santa Cruz Biotechnology) was diluted 1:500 in 10% NGS. Phosphorylated Forkhead box O1 (FOXO1; Ser 256) was diluted 1:300 in 10% NGS (catalog no. ab131339; Abcam).

Fertility trials

Male and female mice were paired when they were approximately 2–4 months of age and continued until they were 6 to 8 months of age. Control, Het, and KO male mice were all paired

with control female mice of proven fertility. The time from when males and females were placed together in cages to the time of parturition was measured in days. We also determined the number of pups per litter. We began the fertility study with 12 pairs each control × control, Het × control, and KO × control. However, after the fertility study, PCR analysis of *Cre* in the testis was conducted and, if *Cre* was expressed in other organs as well, animals were removed from the analysis. This inappropriate *Cre* expression led to reduced numbers of Het × control and KO × control pairs for analysis.

Immunofluorescence

Testes were processed for immunofluorescence similarly to the previously mentioned IHC with the exception of using PBS with 0.05% Tween 20 (catalog no. BP337–100; Fisher Scientific) added. Two primary antibodies were used: rabbit antibody to CD31 (PECAM; catalog no. ab28364; Abcam) and a goat antibody to VE-CADHERIN (catalog no. sc-6458; Santa Cruz Biotechnology). Both were diluted 1:50 in 3% BSA (catalog no. A3311; Sigma-Aldrich) in PBS-Tween. An antirabbit FITC secondary antibody (Alexa Fluor 488; catalog no. 4412S; Cell Signaling Technology) was added to bind the CD31 primary antibody. To detect expression of VE-CADHERIN, we used an antigoat Texas Red secondary antibody (Alexa Fluor 594; catalog no. A-21223; Invitrogen/Life Technologies). Each of the secondary antibodies was diluted 1:400 in 3% BSA in PBS-Tween. Once each of the primary antibodies was optimized with their corresponding secondary antibodies, testes were dual labeled with a mixture of both primary antibodies followed by a mixture of both secondary antibodies. Staining was moved to a dark room just before the fluorescent secondary antibodies were added. Following this incubation and washing in PBS-Tween three times, slides were mounted with VECTASHIELD HardSet Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; catalog no. H-1500; Vector Laboratories) and cover-slipped. Slides were imaged in a dark room using SlideBook 4.2 Software (Intelligent Imaging Innovations) and using an IX71 Olympus Inverted Brightfield and Fluorescence Microscope (Hitschfel Instruments). In addition to sections with all antibodies added, slides had two more isolated sections: one without any antibodies and one with only secondary antibodies added. These negative controls confirmed how much fluorescence was background. Two images were taken at ×100 and ×400 as well as three images at ×200 of both control (n = 5) and KO (n = 4) testes.

Determination of undifferentiated spermatogonia and sperm numbers

Testis sections used for ZBTB16 immunohistochemistry were examined at ×200 magnification with an Olympus BX51 Microscope (Olympus America), and images were captured using cellSens digital imaging software (Olympus America). The number of undifferentiated spermatogonia per tubule was identified as being positive for ZBTB16 staining in each microscopic field and was determined from four randomly chosen, nonoverlapping fields from the center section of each testis per animal. The number of positively stained spermatogonia was averaged per testis, and this average was compared between KO (n = 7) and controls (n = 6). Hematoxylin and eosin-stained epididymal sections were examined at ×400 using the same camera and software. The number of spermatozoa within the corpus epididymis per tubule for each microscopic field was determined from five randomly chosen fields per epididymis per animal. The number of spermatozoa from these five microscopic fields was averaged per tubule,

and this average was compared between KO (n = 5) and control (n = 4) mice. For the same animals and images, we measured tubule area in the corpus epididymides using ImageJ software (National Institutes of Health).

Quantitative RT-PCR (qPCR)

One testis was collected from each animal for RNA extraction, RT to cDNA and qPCR analysis according to previous protocols (19). RNA samples were stored at −80°C until RT was performed using the SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was performed to determine residual genomic DNA. Samples were stored at −20°C until used for qPCR. Equivalent amounts of cDNA were added to master mixes for qPCR. TaqMan Universal PCR and Power SYBR Green Master Mixes were used (Applied Biosystems). Reactions used 150- or 300-nm primers depending on validation, and TaqMan reactions required 2-μM probe. Primers were designed with Primer Express 3.0 software (Applied Biosystems) and synthesized at Integrated DNA Technologies unless otherwise stated. Genes that required SYBR Green to measure quantification were also plotted on a dissociation curve to ensure primer dimerization was not detected. qPCR was conducted according to previous protocols (19). TaqMan Gene Expression Assays were used to detect expression of four genes: glial cell line derived neurotrophic factor (*Gdnf*), ret proto-oncogene (*Ret*), SIN3A (*Sin3a*), and neurogenin 3 (*Neurog3*) (catalog no. 4331182; Applied Biosystems). Probes were designed in our laboratory and purchased for two genes: BCL2-associated X protein (*Bax*) and B-cell leukemia/lymphoma 2 (*Bcl2*) (Applied Biosystems). Primer and probe sequences for each gene are listed in Supplemental Table 1. mRNA abundance was normalized to expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and represented as fold changes relative to the mean control value (set at a value of 1). The expression of *Gapdh* was determined using a VIC probe and primer kit (catalog no. 4308313; Taqman Rodent GapDH Control Reagents; Applied Biosystems). Values were expressed as means ± SEM.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay

Middle sections of control and KO testes were assayed for apoptosis according to a previously devised protocol (catalog no. G3250; Promega). Slides were mounted using a VectaStain mount with DAPI (Vector Laboratories). Testes were imaged in a dark room using SlideBook 4.2 Software (Intelligent Imaging Innovations) and using an IX71 Olympus Inverted Brightfield and Fluorescence Microscope (Hitschfel Instruments). TUNEL-positive cells fluoresced bright green (FITC filter, 488 nm), whereas nuclei shone bright blue because of the DAPI (360–460 nm). Because of no observable differences in apoptosis between KO and control at ×100 magnification, we did not conduct further analysis.

Statistical analysis

Data were analyzed using a one-way ANOVA with a Dunnett's test to compare KO to controls (JMP software; SAS Institute). Differences in data were considered statistically significant when $P < .05$. Fertility data were analyzed with JMP software (SAS Institute) with one-way ANOVA, and the treatment means were compared with a Student *t* test. Differences were considered

significant at $P < .05$. Trends toward significance were considered if $.05 > P < .1$. All treatments were compared relative to controls.

Results

Validity of pDmrt1-Cre;Vegfa$^{-/-}$ KO phenotype

Immunohistochemistry with a pan-VEGFA antibody that detects both angiogenic and antiangiogenic isoforms was used to determine how deletion of *Vegfa* isoforms in Sertoli and germ cells affected protein expression of VEGFA isoforms in those cell types. There was a reduction of VEGFA isoforms in Het (Figure 1B) and KO testes (Figure 1C) vs controls (Figure 1A), especially in Sertoli cells within the seminiferous tubules. To determine global reductions in of VEGFA expression in seminiferous tubules, we used a chromaffin assay in our IHC to detect protein expression of all VEGFA isoforms, both pro- and antiangiogenic. The KO mouse testes had reduced expression of VEGFA isoforms in Sertoli cells and, thus, provide a valid model for examining reduced VEGFA expression in the testis. Sections processed without primary antibody lacked positive staining and served as negative controls (Figure 1D).

Fertility of pDmrt1-Cre;Vegfa$^{-/-}$ and heterozygote male mice

Mice were either mated: 1) male control to female control (Control × Control; n = 12); 2) male KO to female control (KO × Control; n = 4); or 3) male Het to female control (Het × Control; n = 5). The number of days from the initial pairing of males with females to their first parturition was 14 days longer when male KO × control female matings (38.0 ± 11.8 d; $P < .01$) were compared with control matings (24.1 ± 1.4 d; Figure 1E). Furthermore, when all parturition intervals were analyzed, the KO male × control female matings tended to take 9 days longer to get females pregnant when compared with control matings (33.5 ± 4.07 vs 24.5 ± 2.49 days; $P < .07$; Figure 1F). However, there were no differences in Het × Control matings at the first parturition. When we continued to evaluate the Het × Control matings, interestingly, the time from the first to the second parturition in the control females mated with the Het males was 10 days longer than control matings (36.3 ± 2.7 vs 25.8 ± 3.4 d; $P < .05$). Surprisingly, we also observed a difference in the number of pups per litter in the Het × Control matings with a two-pup reduction compared with control matings (6.50 ± 0.65 vs 8.67 ± 0.75; $P < .03$; Figure 1G). However, there were no differences in number of pups between KO × Control and Control × Control matings.

Vasculature in the testicular interstitium appeared unaltered in KO males, but increased mitotic germ cells were different

The presence of undifferentiated spermatogonia has been demonstrated to be increased in portions of seminiferous tubules that border interstitium and especially near vasculature (20). As a result, we performed immunofluorescence to dual label for two known endothelial cell markers—platelet cell adhesion molecule (PECAM-1 or CD31) and vascular endothelial-cadherin (VE-CAD). Although our findings showed no apparent differences in testicular vasculature in KO mice (Figure 2, J–L) compared with the testes of controls (Figure 2, G–I), CD31-positive staining (green) was much more evident in germ cells in the testes of KO males

(Figure 2, D–F) that was not evident in any of the controls (Figure 2, A–C). Serial sections that were processed without primary antibodies were used as negative controls (Figure 2, M–O).

Epididymal morphology was affected in pDmrt1-Cre;Vegfa$^{-/-}$ mice with reduced corpus epididymal sperm numbers per tubule

There were alterations in morphology within the epididymis. The epididymis is important because it is necessary for sperm cell maturation and capacitation. Morphologically, the epididymides in KO mice appeared to have fewer tubules and less coiling than control epididymides (Figure 3, A–F) with greater segmentation between different sections of the organ. Epididymides of KO mice were 23% smaller than controls (0.030 ± 0.0026 vs 0.039 ± 0.0022 g, $P < .05$; Figure 3G). In addition to the reduction in tubule numbers and epididymides weight, the corpus epididymides in KO mice (28.07 ± 4.62; $P < .002$) also had 42% fewer sperm per tubule compared with controls (48.45 ± 3.38; Figure 3H). Interestingly, the tubule area in the corpus epididymides was significantly greater in the KO males (113 9491 ± 18 498 pixels; $P < .03$) compared with controls (106 3001 ± 28 039 pixels; data not shown). In addition, although tubule number did not differ between KO and control males, sperm numbers were still reduced in KO males when not represented per tubule (189.8 ± 30.8; $P = .0003$) compared with control males (357.2 ± 28.4; data not shown).

Alterations in testis morphogenesis in KO and Het mice compared with controls

Even though there was not a significant difference in testis weight, the seminiferous epithelium of the testis was disorganized, with large vacuoles where germ cells normally reside in KO and Het mice (Figure 4, A–F). In some cases, certain staged germ cells seemed to be located in abnormal locations within tubules. We observed more of these morphologic alterations and seminiferous disorganization within Het mice than KO mice testes.

Testicular expression of ZBTB16 protein

To determine the effect of VEGFA loss on populations of spermatogonia, we performed immunohistochemistry for the undifferentiated spermatogonia marker ZBTB16 (also known as PLZF). ZBTB16 is thought to be important for the self-renewal of undifferentiated spermatogonia (21). The number of ZBTB16-positive undifferentiated spermatogonia per tubule was decreased in KO testes (4.38 ± 0.29; $P < .003$; Figure 5B) compared with controls (6.60 ± 0.67; Figure 5A), demonstrating a loss of undifferentiated spermatogonia in KO mice at 6 months of age. Serial sections that were processed without primary antibody served as negative controls (not shown).

Genes critical for SSC niche establishment and maintenance were altered in KO mice testes with reductions in undifferentiated spermatogonia

Within the developing population of undifferentiated spermatogonia, there are several genes that are important in undifferentiated spermatogonia (and possibly SSC) maintenance. One of those factors, RET, is the receptor for the growth factor GDNF that, in the testis, is secreted by Sertoli cells. GDNF-mediated RET signaling is important for the development of undifferentiated

spermatogonia (22, 23). The relative amount of *Ret* mRNA in testes from KO mice was greater (3.53 ± 0.81; $P = .0005$) than that of controls (1 ± 0.16; Figure 6B). However, relative amounts of *Gdnf* mRNA in KO testes were not different from controls (Figure 6A). NEUROG3 is a transcription factor that is expressed in undifferentiated spermatogonia and those that differentiate and ultimately give rise to spermatogenesis (24). The relative amount of *Neurog3* mRNA in KO testes was significantly higher (1.67 ± 0.17; $P < .007$) than control testes (1 ± 0.14; Figure 6D). There was no significant difference in the amount of *Zbtb16* mRNA between KO and control testes (Figure 6C).

SIN3A, as a Sertoli cell product, affects spermatid elongation and is required by Sertoli cells to establish a niche for undifferentiated spermatogonia because it appears to aid in spermatogonial migration to the basement membrane (22). Relative amounts of *Sin3a* mRNA in KO testes tended to be higher (1.82 ± 0.53; $P < .08$) than in controls (1 ± 0.15; Figure 6F). Kit ligand (KITL) has been reported to be required for the survival and proliferation of germ cells and spermatogonia in the testes (25). However, there was no difference between KO and control testes for *Kitl* mRNA abundance (Figure 6E).

Genes critical for regulating cell apoptosis were altered in KO mice testes

Because VEGFA proangiogenic isoform treatment has been demonstrated to increase the expression of *BCL2* relative to *BAX* in bovine testis (26), we evaluated *Bcl2* and *Bax* mRNA expression in the conditional KO vs control testis. Levels for *Bcl2* mRNA in KO testes were higher (2.38 ± 0.66; $P < .02$) than those in control testes (1 ± 0.14; Figure 7A). However, there was no difference in mRNA abundance for *Bax* in KO testes compared with controls (Figure 7B). Furthermore, the ratio of *Bcl2* to *Bax* mRNA levels in mice with Sertoli-germ-cell-specific VEGFA loss was increased (2.24 ± 0.61; $P < .02$) compared with controls (1 ± 0.16; Figure 7C). In addition, mRNA for Caspase 3 (*Casp3*), a factor that promotes cell apoptosis, was not different between KO and control testes (Figure 7D).

Phosphorylated FOXO1 immunohistochemistry is more cytoplasmic in KO mouse testes

FOXO1 is a transcription factor that has been suggested to be important for either self-renewal or differentiation of undifferentiated spermatogonia depending on whether it is phosphorylated (27). Phosphorylated FOXO1-positive staining appeared less intense and more cytoplasmic in KO testes (Figure 8B) when compared with controls (Figure 8A). Sections processed without primary antibody lacked positive staining and served as negative controls (Figure 8C). In addition, the mRNA abundance of total *Foxo1* tended to be reduced (57%) in the testes of KO mice (0.43 ± 0.16; $P < .07$) compared with control mice (1 ± 0.18; Figure 8D).

Body weight was reduced in KO mice

In KO mice, body weight (28.52 ± 0.48 vs 32.52 ± 1.20 g, $P < .02$; Supplemental Figure 2F) was reduced 12.3% compared with those from control mice. In addition, the prostate tended to be 55% smaller (0.01 ± 0.0031 g vs 0.022 ± 0.0045 g; $P < .07$; Supplemental Figure 2B). Although reductions in body weight were observed in comparison to controls, no differences in testis,

seminal vesicles, kidneys, and adrenal gland weights were detected (Supplemental Figure 2, A and C–F). Plasma testosterone concentrations were also not different compared with control mice (Supplemental Figure 2G) at 6 months of age when the mice were collected.

Discussion

In the present study, we present novel data that VEGFA isoforms secreted by Sertoli cells and germ cells are necessary for maintenance of undifferentiated spermatogonia, sperm numbers, and normal male fertility. Again, the *pDmrt-1-Cre* transgene was detected in mouse Sertoli cells and in some germ cells; however, endogenous mouse DMRT-1 does not appear to be translated in germ cells (17). Elimination of all VEGFA isoforms resulted in subfertility, with longer intervals to get females pregnant or achieve viable offspring, and this occurred even when one allele was conditionally knocked out. Furthermore, these data support a recent publication by our laboratory and collaborators that treatment with either proangiogenic or antiangiogenic VEGFA isoforms affect genes regulating the SSC niche, presumably by altering the ability for them to renew or differentiate (6).

In KO testes, the expression of genes that are thought to regulate either renewal or differentiation of undifferentiated spermatogonia was altered. Genes expressed in undifferentiated spermatogonia (*Ret*), in both undifferentiated and early differentiated (*Neurog3*) and in Sertoli cells (*Sin3a*), had greater mRNA abundance (or tended to) compared with controls. NEUROG3 is expressed in most if not all undifferentiated spermatogonia and all those that will give rise to spermatogenesis (24). Loss of both RET and SIN3A causes SSC depletion in mice (22, 23), and inhibition of GDNF signal transduction through RET dramatically impairs SSC renewal (28).

In addition, total *Foxo1* mRNA tended to be reduced in the testes of KO males, and phosphorylated FOXO1 appears to be more cytoplasmic and less intensely expressed. FOXO1 belongs to a family of transcription factors known for their role in regulating cellular processes including cell fate decisions (29, 30). Phosphorylation of FOXO1 results in translocation from the nucleus to the cytoplasm (31). A study involving mice where *Foxo1* was conditionally knocked out in germ cells suggested a novel role for FOXO1 in SSC homeostasis (27). Its nuclear translocation coincided with the initiation of differentiation (expression of *c-kit*), whereas cytoplasmic, phosphorylated expression was associated with self-renewal. It was determined that FOXO1 may regulate SSC renewal through its positive relationship with RET (27). Also, VEGFA_164 has been demonstrated to up-regulate expression of GDNF and to phosphorylate RET in the mouse kidney (32).

Although SIN3A is expressed by Sertoli cells and has been determined to be important for the establishment of the SSC niche (22), it has also been shown to cause sterility when knocked out in germ cells (33). SIN3A functions as a transcriptional corepressor, and one of its targets is ZBTB16 (34). Although mRNA abundance for *Sin3a* was increased in KO male testes in this study, positive staining for ZBTB16 was reduced. In addition, SIN3A appears to be important for indirectly regulating differentiation of germ cells. When the *Zbtb16* gene is knocked out in germ cells, the cells undergo apoptosis as they resume quiescence perinatally (33). The reduction in ZBTB16-positive spermatogonia suggests that loss of VEGFA isoforms may deplete the SSC pool. This supports previous research where injections of perinatal mice (P3–5) with either an

antibody to the proangiogenic isoform, VEGFA_164, or treatment with the antiangiogenic isoform, VEGFA_165B, followed by spermatogonial transplantation at P8 and P21 decreased SSC colonization in testes compared with controls. Combined with the data from this study, it appears that the VEGFA_164 proangiogenic isoform may be critical for sustained SSC renewal, proliferation, or survival that is necessary to maintain the SSC niche, whereas the antiangiogenic isoform, VEGFA_165B, may promote differentiation of germ cells or their demise through apoptosis (6).

Within the testes of KO mice, there were also differences in expression of genes that promote survival: increased *Bcl2* and *Bcl2:Bax* mRNA abundance. In other studies, recombinant mouse VEGFA_164 treatment increased numbers of germ cells in cultured bovine testis explants compared with controls. Furthermore, blocking VEGFA activity in vitro reduces the number of germ cells in VEGFA-treated testis tissue (26). This suggests that proangiogenic VEGFA isoforms are survival factors for germ cells and compensatory mechanisms may exist to enhance prosurvival genes such as *Bcl2*.

With the absence of VEGFA isoforms, it seems the testis was attempting to increase the transcription of factors to maintain the SSC niche (*Sin3a*), possibly renewal through enhanced GDNF signal transduction (*Ret*) and cell survival (*Bcl2* and *Bcl2:Bax*). Because NEUROG3 is expressed in at least a majority of undifferentiated spermatogonia, its increased mRNA abundance could also be indicative of an attempt to maintain the niche. The mRNA abundance for total *Foxo1* tended to be reduced and the positive staining for the phosphorylated FOXO1 appeared less intense in testes of KO males; however, the staining also appeared more cytoplasmic. This is also suggestive of an attempt at compensating for VEGFA isoform loss. In addition, *Casp3* mRNA abundance was not different, and TUNEL staining was not different between KO and control testes (data not shown), further suggestive of protective mechanisms to maintain germ-cell survival. Because previous data suggest that antiangiogenic VEGFA isoforms promote differentiation (6), their absence was presumably driving compensatory mechanisms as well (increased *Neurog3*). The increase in survival factors may have been to maintain germ cells at multiple stages seeing that there were both mature spermatozoa present in the epididymis as well as undifferentiated spermatogonia in the testes. Compensatory mechanisms were able to maintain fertility in the short term, but loss of VEGFA isoforms appears to cause a gradual reduction in fertility as parity increases as demonstrated by the heterozygous males.

In addition to alterations in factors that regulate undifferentiated spermatogonia and survival, body weight was reduced by 12%. The reason for the decrease in body weight in the current study is unknown; however, it is possible that alteration of factors within the testis may have affected body growth and also may have contributed to differences in morphology and size of the prostate. Furthermore, factors produced by the testis have been demonstrated not only to increase body weight and growth but also to affect morphogenesis of the epididymis and potentially maintenance of its function (35).

Although testis weight was not different, vacuoles were visible where germ cells should reside in Het and KO testes compared with controls. Previously, constitutive expression of VEGFA_121 under the control of the mucin 1 (*Muc1*) promoter resulted in subfertility in mice. Seminiferous tubules were disorganized, and spermatogenesis appeared arrested in some of these mice (7). In

another study, overexpression of human VEGFA_165 in the testis and epididymis under the control of the mouse mammary tumor virus promoter resulted in infertility through spermatogenic arrest and endothelial cell hyperplasia (36).

Both Sertoli and germ cells secrete VEGFA, and it is involved in the production of plasmin (urokinase plasminogen activator or uPA and tissue plasminogen activator or tPA) and plasmin inhibitors (plasminogen activator inhibitor-1 or PAI-1), which may alter cell adhesion and the ability for germ cells to migrate from the adluminal to luminal compartment through the blood-testis barrier (37–40). Loss of VEGFA isoforms may result in reduced regulation of movement as well as in the epithelial disorganization similar to what was seen when VEGFA_121 was under control of either the *Muc1* or mouse mammary tumor virus promoters (7, 36). Furthermore, the loss of undifferentiated spermatogonia could be impairing communication between Sertoli and germ cells, complicating the already disconnected communication between Sertoli and germ cells.

In an attempt to elucidate whether the effects of VEGFA elimination in this study were due to vascular alterations to the niche and subsequently the previously mentioned weakening of Sertoli-germ-cell adhesion, we examined the expression of CD31 (PECAM-1) and VE-CAD. Testicular vasculature was dual-labeled fluorescently for both proteins and was not dramatically different between any KO and control testes. Although both endothelial cell markers appeared to favor the interstitial compartment and vasculature, CD31-positive staining was seen in germ cells in the KO testes stained and not in the controls. CD31 has been demonstrated to be an important receptor on human sperm to potentially initiate capacitation (41, 42). Also, CD31 has also been shown to label migrating male-specific endothelial cells (43). Finally, although CD31 has been demonstrated to be important for adherence, CD31-positive staining in migratory germ cells suggests that it is required for adherence to Sertoli cells in the testis (42). Expression of VE-CAD did not appear different between KO and control testes; however, it could be seen in the interstitium as well as in what appear to be adherens junctions between Sertoli cells and possibly some germ cells.

Removal of VEGFA isoforms in Sertoli and in subpopulations of germ cells resulted in smaller body, epididymal, and prostate weights, altered expression of testicular genes regulating cell apoptosis, and undifferentiated spermatogonia, and in increased expression of CD31 in germ cells. Furthermore, KO mice had reduced numbers of undifferentiated spermatogonia in the testis and numbers of sperm in the corpus epididymis, which would result in reduced male fertility. Therefore, whether the effects are direct or indirect, the collective results in this study demonstrate that VEGFA is a regulator of genes that are necessary for maintenance of undifferentiated spermatogonia, sperm production, and male fertility.

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Disclosure Summary: The authors have nothing to disclose.

Footnotes

Abbreviations:

DAPI
: 4′,6-diamidino-2-phenylindole

E
: embryonic day

FOXO1
: Forkhead box O1

GDNF
: glial-cell line-derived neurotrophic factor

Het
: heterozygotes

IHC
: immunohistochemistry

KO
: knockout

NGS
: normal goat serum

P
: postnatal day

qPCR
: quantitative RT-PCR

SSC
: spermatogonial stem cell

TUNEL
: terminal deoxynucleotidyl transferase dUTP nick end-labeling

VE-CAD
: vascular endothelial-cadherin

VEGFA
: vascular endothelial growth factor A.

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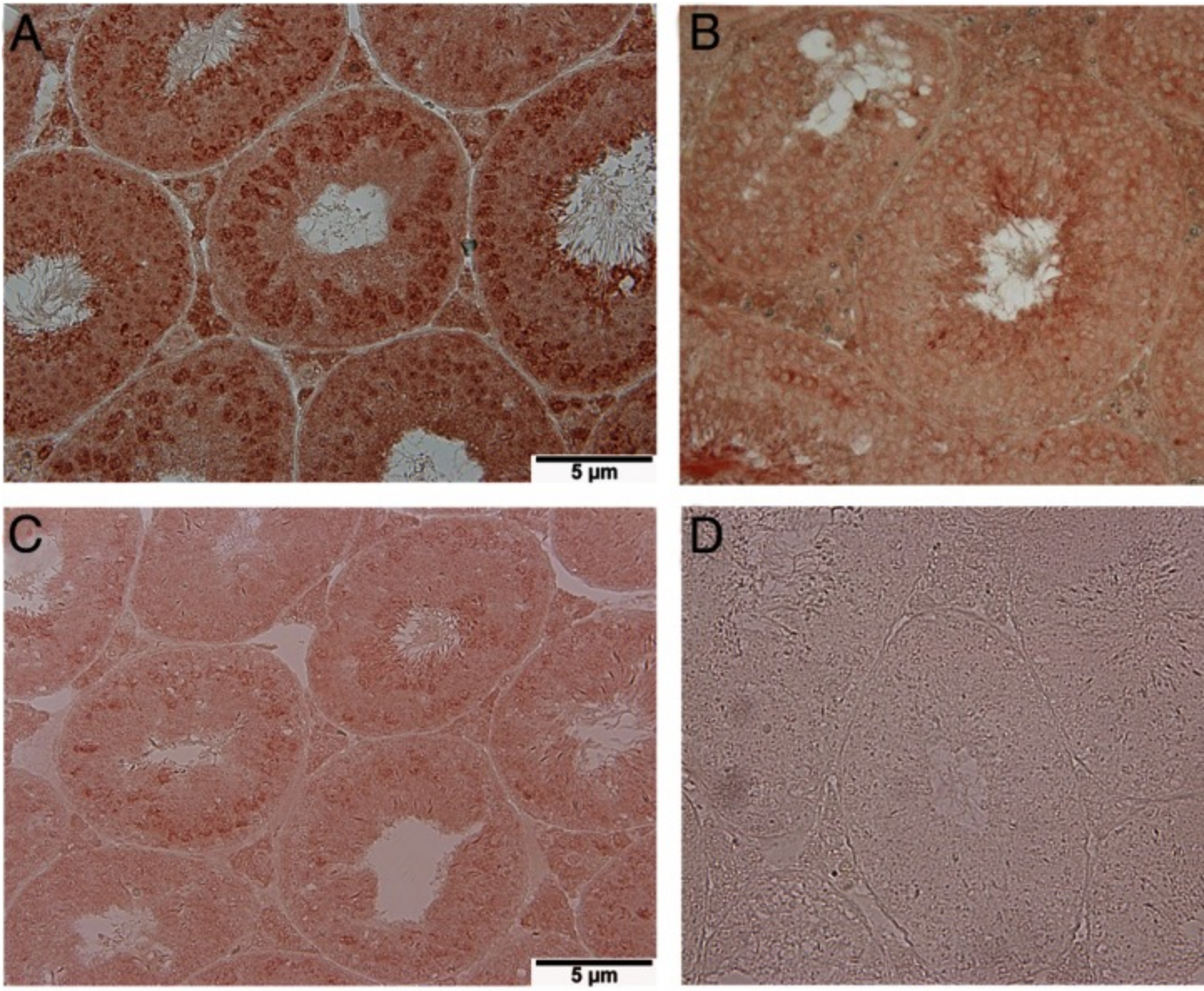
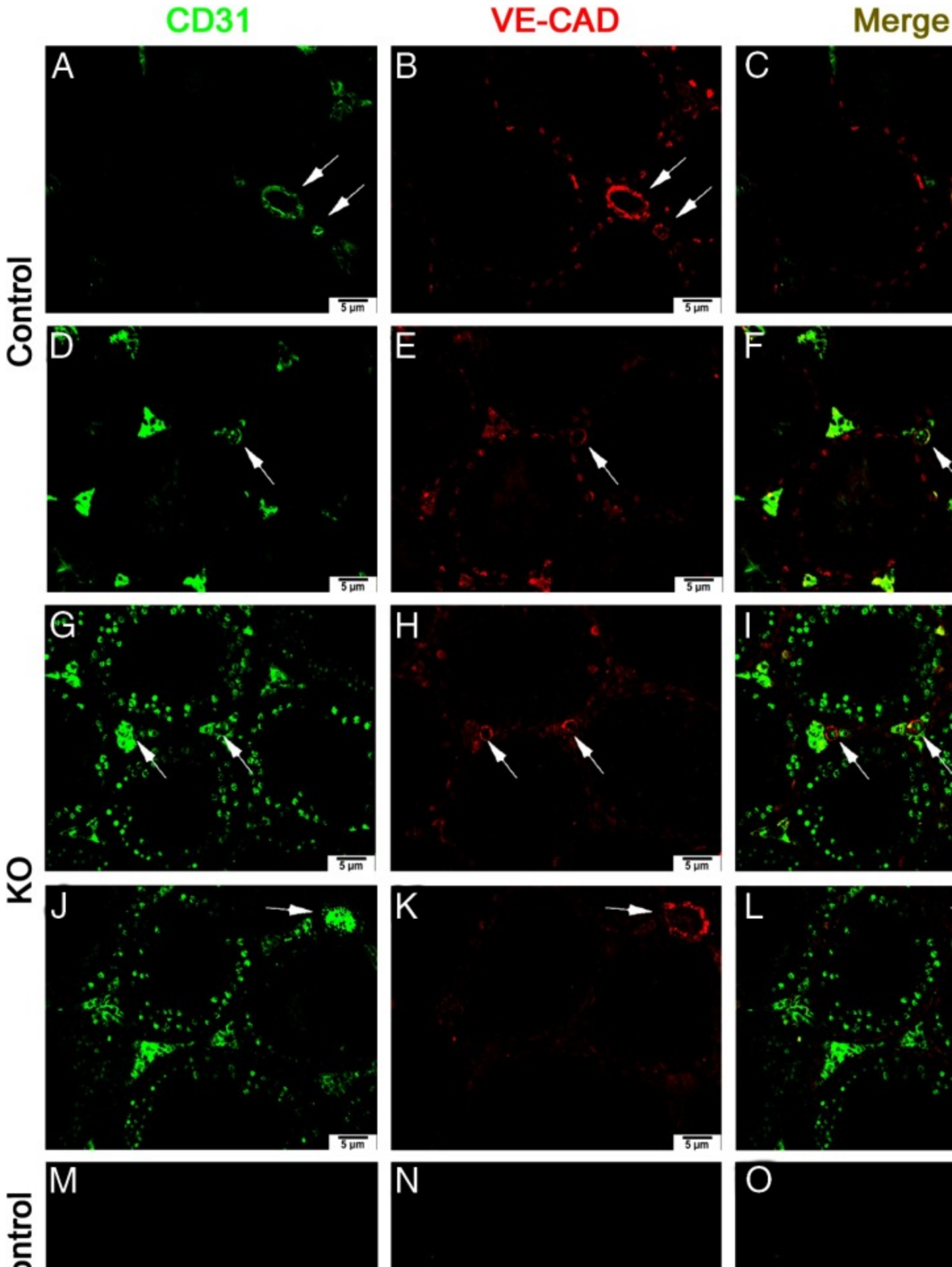
Figures and Tables

Figure 1.


A
5 µm
B
C
5 µm
D
E
P=0.01
Mating to 1st Parturition (days)
60
50
40
30
20
10
0
a
a
b
Cont x Cont
Het x Cont
KO x Cont
F
Parturition Intervals (days)
40
35
30
25
20
15
10
5
0
a
a
Cont x Cont
Het x Cont
KO
G
P=0.03
12
10
a

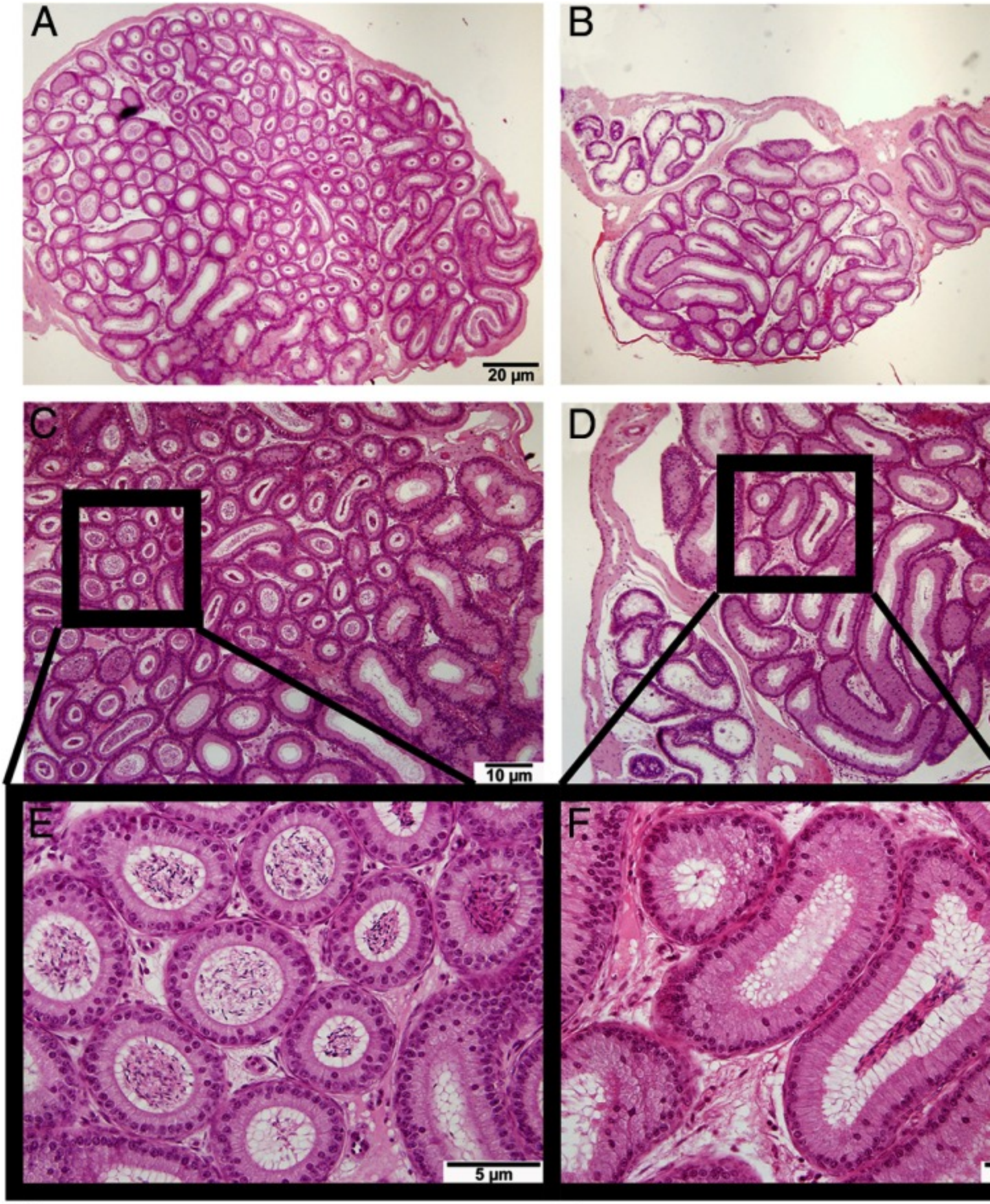
Immunohistochemistry with panVEGFA antibody at ×400 magnification in a control testis (A), heterozygous (Het) testis (B), and a KO testis (C). Negative control testes were processed without primary antibody (D). Results from the fertility trial with males as the first present in each pair (Control, Het, KO) were broken into days from mating to first parturition (E), the average number of days between each parturition interval (F), and average number of pups born per litter (G). For the Control × Control, Het × Control, and KO × Control pairs in (E), n = 12, 5, and 4, respectively. For the Control × Control, Het × Control, and KO × Control pairs in (F) and (G), n = 16, 8 and 6, respectively. Results represented by different letters were significant when $P < .05$; †, data tended to be significant ($.05 < P < .1$).

Figure 2.


CD31
VE-CAD
Merge
Control
KO
Control
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
5 μm

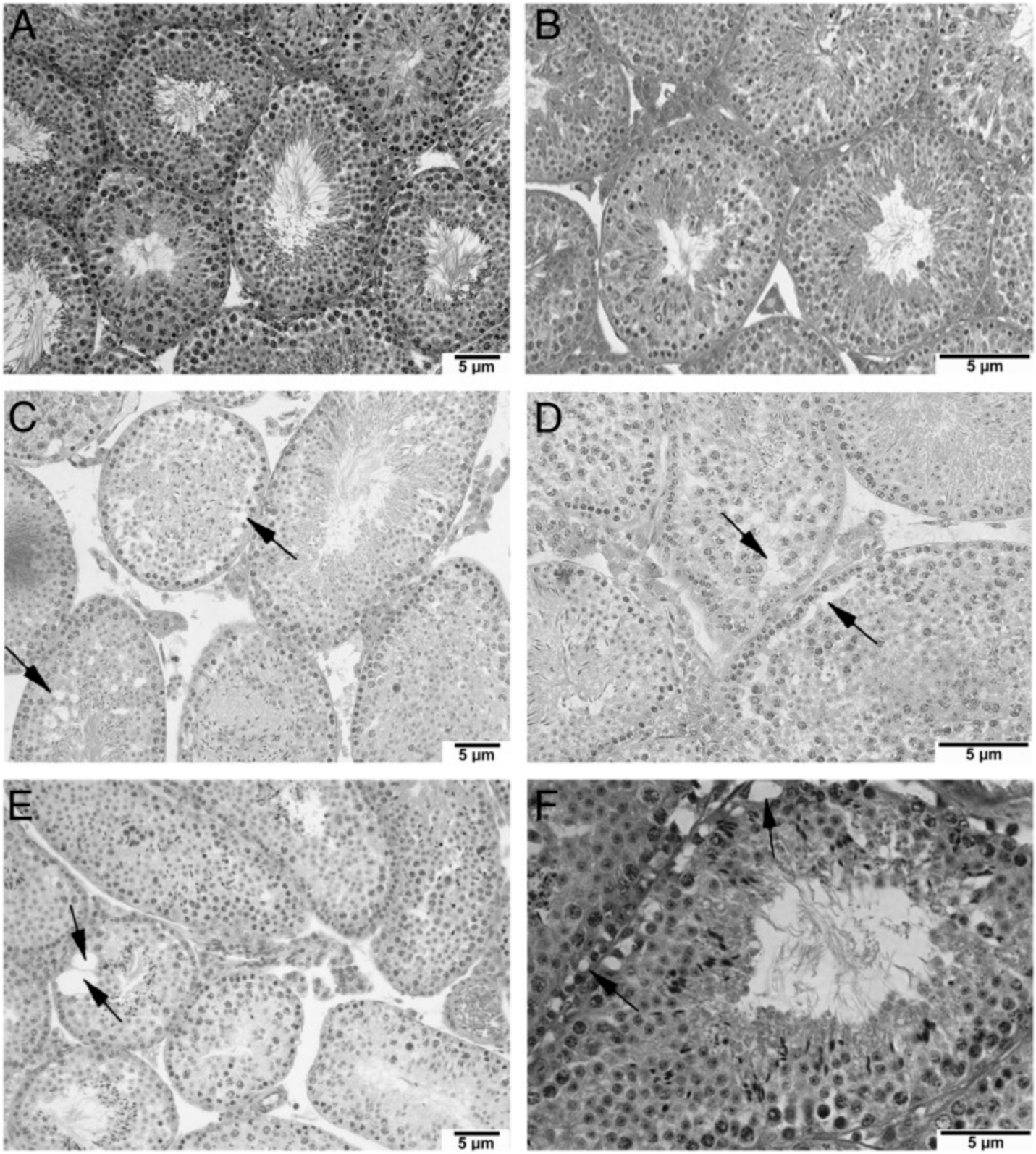
Fluorescent images of mouse testes at ×200 focusing on both tubules and blood vessels in control mice (A–F) and knockout mice (G–L). CD31-positive staining is green (A, D, G, J). VE-CAD-positive staining is red (B, E, H, K). Merging of the two appears yellow where coexpressed (C, F, I, L). Negative controls were processed without primary antibodies. Pictured are negative control images viewed with the FITC filter (M), the Texas Red filter (N), and finally the merge with background reduced (O).

Figure 3.


A
20 µm
B
C
10 µm
D
E
5 µm
F
G
0.045
0.04
0.035
P<0.03
*
H
60
50

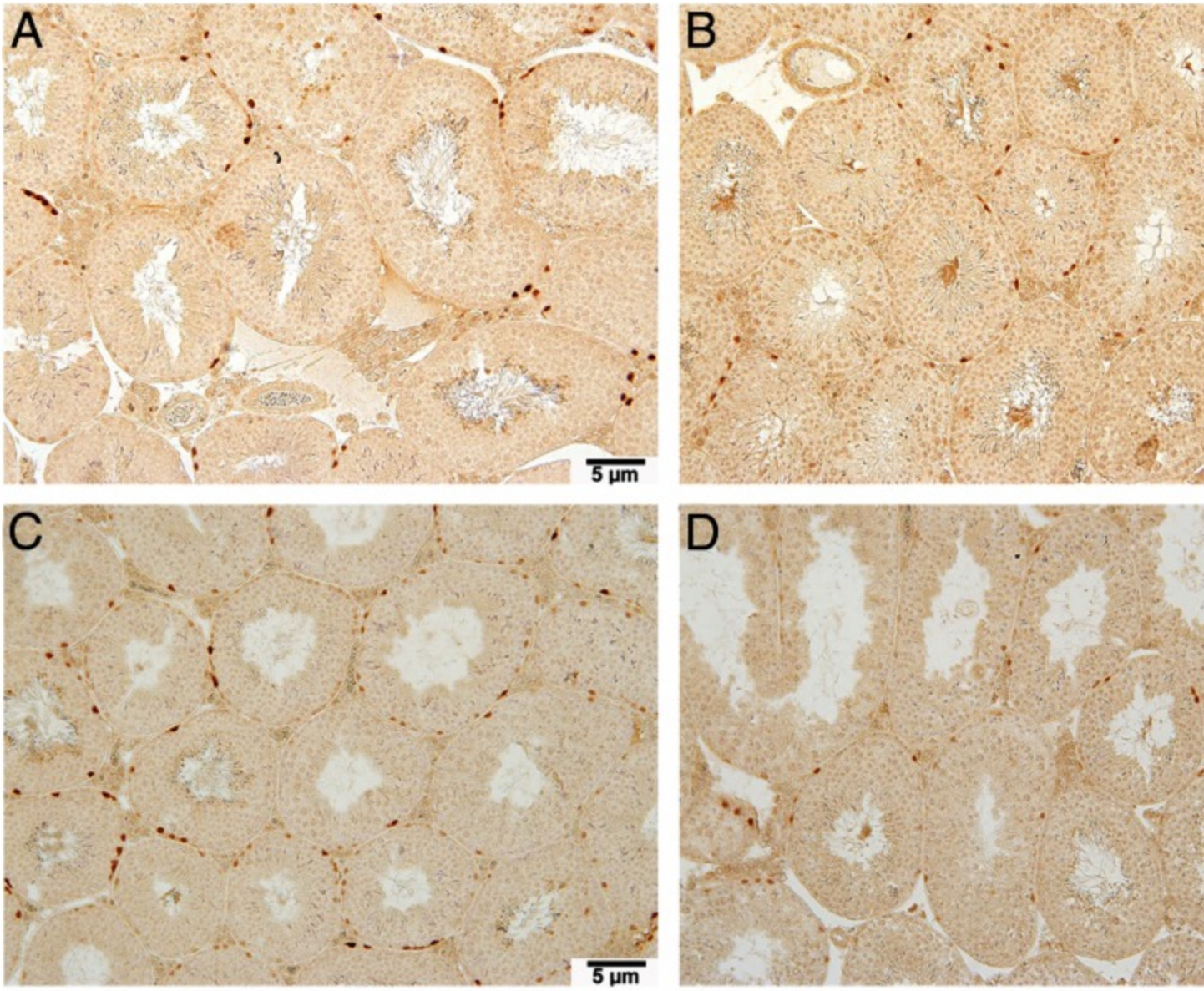
Hematoxylin-eosin staining in the epididymides of controls (A, C) and KOs (B, D) at ×40 and ×100. Inset views of control (E) and KO (F) epididymides are at ×400. The number of spermatozoa per tubule in the corpus epididymis from control (n = 20) and KO males (n = 23) is represented (G). *, Results were significant when $P < .05$.

Figure 4.


A
5 µm
B
5 µm
C
5 µm
D
5 µm
E
5 µm
F
5 µm

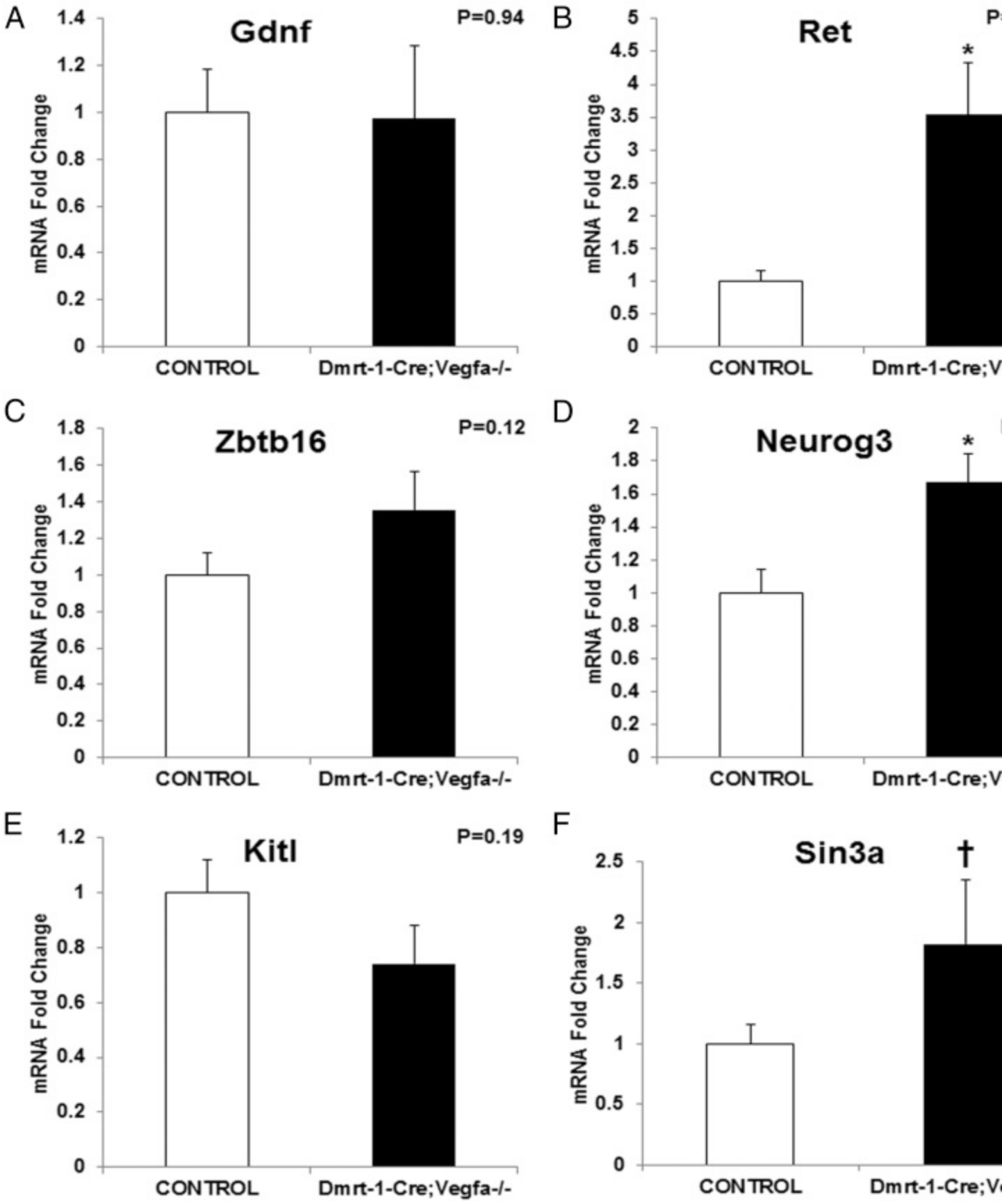
Hematoxylin-eosin staining of testis cross-sections in black and white of ×200 control (A), ×400 control (B), ×200 heterozygote (Het) (C), ×400 Het (D), ×200 KO (E), ×400 KO (F). Arrows denote seminiferous epithelium disruptions.

Figure 5.


A
5 µm
B
C
5 µm
D
E
P<0.003
ZBTB16-Positive Cells Per Tubule
0
1
2
3
4
5
6
7
8
9
*
Control
Dmrt1-Cre;Vegfa-/-

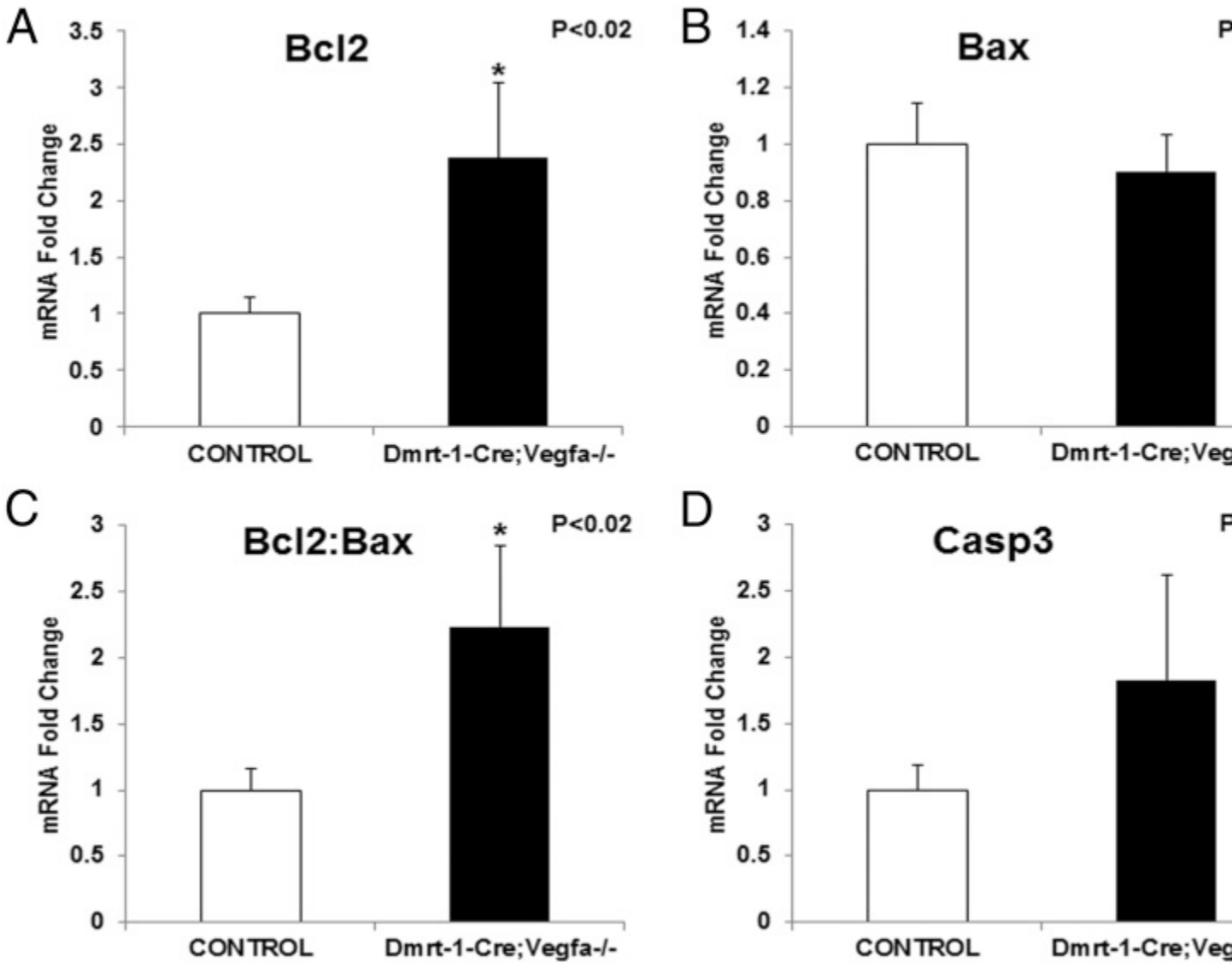
ZBTB16-positive staining at ×200 magnification in cross-sections of control (A and C) and KO (B and D) testes. Undifferentiated spermatogonia that stained positive for ZBTB16 are denoted by red-brown staining. Also represented are the counts of ZBTB16-positive undifferentiated spermatogonia represented per tubule between control (n = 24) and KO (n = 27) testes (E). *, Results were significant when $P < .05$.

Figure 6.


A
Gdnf
P=0.94
mRNA Fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
Dmrt-1-Cre;Vegfa-/-
B
Ret
*
mRNA Fold Change
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CONTROL
Dmrt-1-Cre;V
C
Zbtb16
P=0.12
mRNA Fold Change
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
Dmrt-1-Cre;Vegfa-/-
D
Neurog3
*
mRNA Fold Change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
Dmrt-1-Cre;V
E
Kitl
P=0.19
mRNA Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
Dmrt-1-Cre;Vegfa-/-
F
Sin3a
†
mRNA Fold Change
2.5
2
1.5
1
0.5
0
CONTROL
Dmrt-1-Cre;V

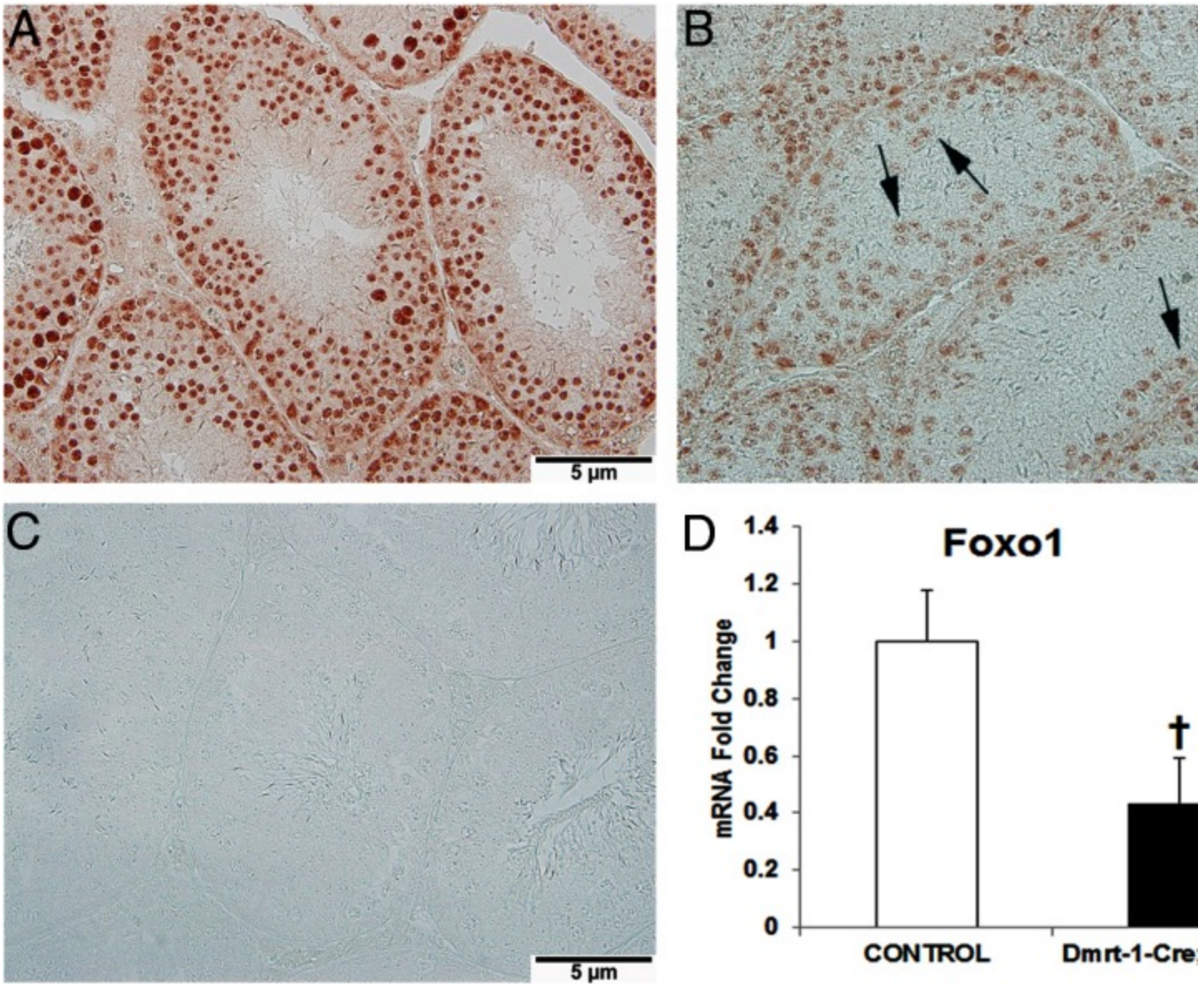
Whole testis mRNA abundance of genes that regulate undifferentiated spermatogonia: *Gdnf* (A), *Ret* (B), *Zbtb16* (C), *Neurog3* (D), *Kitl* (E), and *Sin3a* (F). Mean KO values (n = 8–17) are represented as fold changes ± SEM compared with control (n = 5–9) mean (set to 1). *, Results were significant when $P < .05$; †, data tended to be significant ($.05 < P < .1$).

Figure 7.



Whole testis mRNA abundance of genes that regulate apoptosis and survival: *Bcl2* (A), *Bax* (B), *Bcl2:Bax* (C), and *Casp3* (D). Mean KO values (n = 11–17) are represented as fold changes ± SEM compared with control (n = 5–9) mean (set to 1). *, Results were significant when $P < .05$.

Figure 8.



Immunohistochemistry depicted at ×400 for phosphorylated FOXO1 in the testes of control males (A) and KO males (B). A negative control testis was processed without primary antibody (C). Arrows point to cytoplasmic phosphorylated FOXO1 staining in the KO testis (B). Total *Foxo1* mRNA abundance in the testis was measured and compared between control (n = 11) and KO (n = 5) males (D). †, Results were significant when $0.05 < P < .1$.

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