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VEGFA Family Isoforms Regulate Spermatogonial Stem Cell Homeostasis *in Vivo*

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Abstract

The objective of the present study was to investigate vascular endothelial growth factor A (VEGFA) isoform regulation of cell fate decisions of spermatogonial stem cells (SSC) *in vivo*. The expression pattern and cell-specific distribution of VEGF isoforms, receptors, and coreceptors during testis development postnatal d 1–180 suggest a nonvascular function for VEGF regulation of early germ cell homeostasis. Populations of undifferentiated spermatogonia present shortly after birth were positive for VEGF receptor activation as demonstrated by immunohistochemical analysis. Thus, we hypothesized that proangiogenic isoforms of VEGF (VEGFA₁₆₄) stimulate SSC self-renewal, whereas antiangiogenic isoforms of VEGF (VEGFA_{165b}) induce differentiation of SSC. To test this hypothesis, we used transplantation to assay the stem cell activity of SSC obtained from neonatal mice treated daily from postnatal d 3–5 with 1) vehicle, 2) VEGFA₁₆₄, 3) VEGFA_{165b}, 4) IgG control, 5) anti-VEGFA₁₆₄, and 6) anti-VEGFA_{165b}. SSC transplantation analysis demonstrated that VEGFA₁₆₄ supports self-renewal, whereas VEGFA_{165b} stimulates differentiation of mouse SSC *in vivo*. Gene expression analysis

of SSC-associated factors and morphometric analysis of germ cell populations confirmed the effects of treatment on modulating the biological activity of SSC. These findings indicate a nonvascular role for VEGF in testis development and suggest that a delicate balance between VEGFA₁₆₄ and VEGFA_{165b} isoforms orchestrates the cell fate decisions of SSC. Future *in vivo* and *in vitro* experimentation will focus on elucidating the mechanisms by which VEGFA isoforms regulate SSC homeostasis.

Spermatogonial stem cells (SSC) reside in the seminiferous tubules of the testis and are the only adult stem cell population capable of transmitting genes to offspring. SSC and their ability to self-renew ensure maintenance of fertility throughout the lifespan of the male, whereas SSC differentiation functions to provide germ cell progeny leading to the production of spermatozoa. The process, spermatogenesis, is one of the most productive biological processes in mammals, leading to a virtually unlimited supply of spermatozoa throughout the lifespan of an adult male. Thus, the balance between SSC self-renewal and differentiation, or homeostasis, is critical for male fertility.

In rodents, gonocytes are the first male-specific germ cells present in the testis during fetal and postnatal development (1), and these cells undergo mitotic arrest late in gestation until shortly after birth when they resume mitosis around postnatal d 1.5 (P1.5) to P3 (2). Gonocytes ultimately give rise to the SSC pool but must first migrate from the center of the testicular cords to the basement membrane, an event initiated around P3 (3). During this period of migration, gonocytes resume mitosis (4, 5) and undergo one of three fates: some mature into SSC, some differentiate and initiate spermatogenesis, whereas the remainder degenerate (4–6). *In vivo* transplantation studies indicate that testicular germ cells obtained from P0–P3 mice can colonize recipient testes but do not initiate self-renewal or establish donor-derived spermatogenesis (1, 6). In contrast, germ cells collected from P4–P5 testes generate large areas of donor-derived spermatogenesis in recipients after colonization (7), indicating the presence of robust stem cell activity and SSC formation.

SSC reside near the basement membrane of seminiferous tubules and are intimately associated with somatic cells that include Sertoli cells, peritubular myoid cells, and Leydig cells. Accordingly, the environmental cues influencing the cell fate decisions of SSC during this critical period of germ cell development remain unclear, but it is thought that intrinsic and extrinsic factors produced by the stem cell niche may function to modulate SSC homeostasis *in vivo* (8). For example, glial cell line-derived neurotrophic factor (GDNF) produced by Sertoli cells functions to directly regulate the maintenance and self-renewal of SSC (9), a result confirmed by transplantation (10). However, identification of the additional factors regulating SSC remains difficult because no *bona fide* SSC markers exist, and the only way stem cell activity can be determined is with use of the transplantation assay (11).

Several reports suggest that blood vessels in the interstitial compartment of the testis constitute a niche microenvironment for undifferentiated spermatogonia and SSC populations in the postnatal testis (12–14). Along those lines, angiogenic factors such as vascular endothelial growth factor A (VEGFA) and its receptors have been implicated in nonvascular roles such as directing testis morphogenesis in mice (15, 16) and regulating the biological activity of undifferentiated germ cells in bovine testis tissue (17) in a manner independent of angiogenesis (17, 18). These studies

have yet to 1) determine the function of VEGF family molecules on regulating the cell fate decisions of SSC and 2) differentiate between the effects of proangiogenic isoforms (VEGF_{xxx}) and antiangiogenic (VEGF_{xxx}b) isoforms. Alternative splicing of *VEGFA* yields multiple proangiogenic isoforms (VEGFA₁₁₁, VEGFA₁₂₁, VEGFA₁₄₅, VEGFA₁₆₂, VEGFA₁₆₅, VEGFA₁₈₃, and VEGFA₂₀₆) and multiple antiangiogenic (VEGFA₁₂₁b, VEGFA₁₄₅b, VEGFA₁₆₅b, VEGFA₁₈₃b, and VEGFA₁₈₉b) isoforms (19). VEGFA₁₆₅ (VEGFA₁₆₄ in mice) and VEGFA₁₆₅b are the most prevalent and biologically active splice variants in humans, respectively. The VEGFA splice variants are generated by proximal and distal splice sites in exon 8 of the *VEGFA* gene (19). Moreover, these families of VEGFA isoforms have diverse functions *in vivo*: VEGFA₁₆₅ stimulates endothelial cell proliferation, migration, and survival (20–22), whereas VEGFA₁₆₅b isoforms inhibit VEGFA₁₆₅-induced signal transduction and physiological outcomes (23–29). Three VEGFA receptors (VEGFR) have been identified called FLT1 (VEGFR1), KDR (VEGFR2), and VEGFR3 with kinase insert domain receptor (KDR) eliciting the greatest intracellular signaling for promoting cell proliferation, whereas fms-related tyrosine kinase 1 (FLT1) activation stimulates cell migration; the role of VEGFR3 is presently unknown. In addition to multiple receptors, VEGFA isoforms interact with two soluble cofactors, neuropilin 1 (NRP1) and NRP2, that can either facilitate or suppress VEGFA isoform-receptor interaction and subsequent signaling. Nevertheless, signal transduction events triggered by VEGFA family members within nonvascular targets are poorly understood, especially in the postnatal testis.

The objective of the present study was to evaluate the role of VEGFA isoform signaling *in vivo* in regard to regulating the cell fate decisions of SSC. We hypothesized that proangiogenic isoforms of VEGFA (VEGFA₁₆₄) stimulate SSC self-renewal, whereas antiangiogenic isoforms (VEGFA₁₆₅b) induce differentiation of SSC. To test this hypothesis, we used the functional SSC transplantation technique to assay the effects of VEGFA isoform treatment combinations on the stem cell activity of SSC *in vivo*.

Materials and Methods

Animal care and treatments

All animal experiments were approved by Washington State University Animal Care and Use committees and were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals of the National Institutes of Health. Animals were housed in a standard animal facility and provided *ad libitum* access to food and water. Rosa26 (stock no. 002192) and C57BL/6 (stock no. 000664) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Quantitative real-time RT-PCR (qRT-PCR) analysis

qRT-PCR was conducted as previously described (17) using an iCycler iQ (Bio-Rad, Hercules, CA) detection system. Total RNA was prepared from C57BL/6 testis tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA concentration and purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany), and only samples with a 260/280 ratio of at least 1.8 were used for qRT-PCR analysis. A list and description of TaqMan probe sets used for gene expression in

the current study are provided ([Table 1](#)). Ribosomal protein S2 was used as a normalization reference. Relative quantification of mRNA levels was calculated using the Q-Gene method ([30](#)). At least three donors were used for each age and treatment ($n \geq 3$).

Immunohistochemistry of VEGF ligands and receptors

Immunohistochemistry was performed on paraffin-embedded testis tissue sections (5 μm thickness) obtained from normal C57BL/6 mice using the avidin-biotin complex method and sodium citrate antigen retrieval (10 mM sodium citrate, pH 6.0), as described ([31](#)). A list of affinity-purified primary antibodies (diluted in 10% normal serum, pH 7.4) is provided ([Table 2](#)). Immunoreactivity was detected using 3,3'-diaminobenzidine, and sections were counterstained with hematoxylin. As a negative control, serial sections were processed without primary antibody. At least three donors were used for each age and antigen evaluated ($n \geq 3$).

Analysis of VEGFR activation (phosphorylation) *in vivo*

Testis tissue obtained from neonatal and postnatal C57BL/6 mice was detunicated, fixed in 4% paraformaldehyde (in 10 mM PBS) at 4 C for 8 h followed by paraffin embedding and sectioning (5 μm thickness). After standard processing, heat-induced epitope retrieval with Tris-EDTA buffer [10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20 (pH 9.0)] was used to unmask antigens before quenching endogenous peroxidase activity. After several washes in PBS, slides were blocked (10% normal serum vol/vol in PBS) for 30 min, incubated with affinity-purified antibodies to detect endogenous VEGFR signaling (Abcam, Cambridge, MA) for 12 h at 4 C. Briefly, sections were incubated with 1) rabbit polyclonal to the kinase active domain of human FLT1 (1:150; ab62183) phosphorylated at tyrosine 1333 (V-L-Y^P-S-T) ([32](#)), 2) rabbit polyclonal to activated catalytic domain of human KDR (1:150; ab63405) phosphorylated at tyrosine 1054 (d-I-Y^P-K-D) ([33–35](#)), and 3) rabbit polyclonal to activated human NRP1 (1:100; ab71766) phosphorylated at threonine T916 (LNTQS) ([36, 37](#)). After a series of washes, sections were processed with a biotinylated goat antirabbit secondary antibody for 60 min and then washed again and incubated with streptavidin-horseradish peroxidase substrate for 20 min at 25 C. Immunoreactivity was detected after a 2-min incubation in 3,3'-diaminobenzidine, and sections were lightly counterstained with hematoxylin. Sections of developing mouse kidney were processed as a positive control (data not shown). A negative control, serial sections were processed without primary antibody. Four donors were used for each age and antigen evaluated ($n = 4$).

Neonatal mice treatment

Neonatal mice were treated according to the methodology described by Gerber *et al.* ([38](#)), with minor modifications. Fifty-microliter glass syringes (Hamilton, Reno, NV) were used to administer all ip treatments (10 μl each). Briefly, A 30-gauge needle was inserted approximately 2–3 mm (commensurate with body wall thickness from P3–P5) into the lower right quadrant of the abdominal cavity at a 20° angle (to avoid the cecum and urinary bladder) and ensure rapid delivery of our various treatments. Neonatal mice (P3–P5) received a single, daily ip injection of vehicle (10 mM PBS plus 0.1% BSA), VEGFA₁₆₄ (500 ng, wt/vol; R&D Systems, Minneapolis, MN), or VEGFA_{165b} (500 ng, wt/vol; R&D Systems) on P3, P4, and P5, respectively. To

evaluate the effect of blocking VEGFA isoforms, we also treated neonatal mice with affinity-purified antibodies against VEGFA₁₆₄ (1 µg, wt/vol; Santa Cruz Biotechnology, Santa Cruz, CA), VEGFA_{165b} (1 µg, wt/vol; Abcam), or rabbit IgG (1 µg, wt/vol; Vector Laboratories, Burlingame, CA) within a similar timeframe. After treatment, Rosa26 mice were killed at P8 and P22, and testes were processed to obtain donor SSC for transplantation. In addition, we evaluated testicular growth and kinetics of germ and Sertoli cell proliferation in a group of C57BL/6 mice killed at 3 d (P8) and 17 d (P22) after treatment. Testes were also harvested from a subset of C57BL/6 mice at P8, P12, and P22 to determine the effect of treatments on the mRNA expression of factors associated with the biological activity of SSC.

Preparation of donor germ cells

Rosa26 donor testes were enzymatically digested as described by McLean ([11](#)), with minor modifications. Briefly, testis tissue was transferred to a dish containing digestion medium, which consisted of 0.18 mg/ml trypsin (GibcoBRL, Bethesda, MD) 0.16 mg/ml collagenase type IV (Sigma Chemical Co., St. Louis, MO), and 0.6 mg/ml deoxyribonuclease (Sigma) in MEM α -medium (pH 7.4) and incubated for 10 min at 37 C. After incubation and addition of FBS (10% vol/vol), testis tissue digests were dispersed by gentle pipetting, and the resulting cell suspension was centrifuged at $600 \times g$ for 7 min at 4 C. After several washes, donor cells were resuspended in MEM containing 0.03% trypan blue (GibcoBRL) to obtain spermatogonial stem cells for transplantation at a concentration of 10^7 cells/ml.

Germ cell transplantation and analysis of recipient testes

To determine stem cell activity, approximately 7.8×10^4 donor cells were transplanted into seminiferous tubules of busulfan-treated, immunologically compatible 129SvCP \times C57BL/6 F1 hybrid recipient mice. When the infused volume deviated from 7 µl, the actual cell number injected was used for colonization efficiency calculations. Recipient mice were killed 8 wk after transplantation, and the colonization efficiency of Rosa26 SSC were determined by counting the number and length of blue colonies after staining with 5-bromo-4-chloro-3-indoyl β -d-galactoside.

Statistical analysis

All datasets are presented as the mean \pm sem, and differences between ages and treatment groups were considered significant at $P < 0.05$. The effect of treatments on testis size, germ cell number, expression of SSC niche-associated factors, and stem cell activity were analyzed using a one-way ANOVA, and pairwise comparisons were evaluated with a Newman-Keuls multiple-range test. The effect of age on gene expression during testis development was also evaluated using ANOVA, and homogeneity of variance was determined using the Bartlett's test. Heteroscedastic datasets were analyzed using the Kruskal-Wallis test, and comparisons between groups were evaluated using the Dunn's multiple-comparison *post hoc* test ($P < 0.05$).

Results

Gene expression of VEGF family molecules during mouse testis development

We assayed the expression of VEGF isoforms, receptors, and coreceptors during mouse testis development. The expression of each gene at each age was compared with the expression at P0 (day of birth), which was set at 1.0. This provides an accurate evaluation of gene expression during testis development for interpretation. The mouse ages selected for gene expression analysis coincided with the age when germ cell-initiated biological events occur, including migration and differentiation. Similarly, specific time points for somatic proliferation and differentiation were included to develop a complete understanding of the dynamics of testis development associated with VEGF signaling. *Vegfa* isoform expression was highest during early testis development until declining after P20 to P180 (Fig. 1A). *Flt1* (*vegfr1*) expression was highest at birth and declined until P8, followed by an increase in expression at P10–P12 and then declining at P14 and remaining at this level throughout adulthood. *Kdr* (*vegfr2*) expression was highest during P1–P6, intermediate during P8–P20, and lowest from P20 throughout adulthood (Fig. 1C). *Nrp1* was expressed at a stable level from birth until P20, followed by a 2- and 4-fold decrease at P35 and P105 that remained constant through P180 (Fig. 1D). *Nrp2* was expressed in the neonatal testis from birth until P10 with a sharp increase at P12. *Nrp2* expression subsequently declined 2-, 4-, and 6-fold from P20 until P35, P105, and P180, respectively, compared with its peak at P12 (Fig. 1E). The expression of VEGFA family molecules in the testis is present during the neonatal period concurrent with SSC formation and proliferation. The VEGFA primers did not distinguish between splice variant isoforms; thus, we conducted immunohistochemistry with antibodies specific for VEGFA₁₆₄ and VEGFA_{165b}, the two most biologically active isoforms.

Protein expression of VEGF family molecules during mouse testis development

To determine the specific cells that express the VEGF isoforms, receptors, and coreceptors, we assayed with immunohistochemistry the expression of these proteins daily from birth until P22 in mouse testes. SSC form at P3 and initiate differentiation and proliferation between P5 and P8 (7), so we focused on precise analysis of VEGFA isoform expression in specific testicular cells at these ages. Comparison with negative controls (omission of primary antibody, Fig. 2, A–C) demonstrates that VEGFA₁₆₄ expression was present in gonocytes and to a lesser degree in Sertoli cells at P3 (Fig. 2D) until P5 (Fig. 2E). From P5–P8, the expression of VEGFA₁₆₄ was mainly in Sertoli cells (Fig. 2, E and F) with a marked reduction in germ cell expression compared with the first 5 d of life. This age coincides with gonocyte migration and formation of the SSC population. In contrast, VEGFA_{165b} was more heterogeneous in expression during the P3–P5 period, being restricted to a distinct subset of gonocyte, undifferentiated spermatogonia, and Sertoli cell populations in the seminiferous tubules (Fig. 2, G–I). VEGFA_{165b} was also present in primary spermatocytes and round spermatids at P20 (Supplemental Fig. 1G, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>).

FLT1 (VEGFR1) and KDR (VEGFR2) were remarkably similar in expression pattern within cell types that were positive for each protein. As can be seen in Supplemental Fig. 1, A–D, FLT1 and KDR were expressed in gonocytes at P3, spermatogonia at P5, and Sertoli cells. NRP1 was expressed by gonocytes and Sertoli cells at P3 (Supplemental Fig. 1E) and primarily localized to undifferentiated spermatogonia at P5 (Supplemental Fig. 1F) with continued expression in

spermatogonia from P6–P20. FLT1 and KDR were also detected within testicular interstitial somatic cells throughout development, evidenced by uniform localization in populations of Leydig cells and spasmodic expression in lymphatic endothelial cells (Supplemental Fig. 1, A–D).

VEGFR signaling is active in germ and somatic cells during mouse testis development *in vivo*

VEGFA_{164/165b} isoforms primarily elicit their effects on cell function after binding to KDR and FLT1, although signal transduction is more pronounced after KDR binding compared with FLT1 binding. Activation of KDR occurs via two independent pathways: VEGFA_{164/165b} binds directly to KDR or VEGFA isoforms bind directly to NRP1, which presents the ligand to KDR after heterodimerization. Thus, after characterizing the patterns and cell-specific aspects of VEGF ligand and receptor mRNA and protein expression *in vivo*, we wanted to determine the specific cell types affected by VEGF signaling during neonatal testis development. To accomplish this goal, we used immunohistochemical analysis to detect the phosphorylation-specific expression of p-FLT1^{Y1333}, p-KDR^{Y1054}, and p-NRP1^{T916} in germ and somatic cells during gonocyte maturation (P1–P5), SSC formation (P5–P6), and the first round of SSC expansion and self-renewal *in vivo* (P8–P14).

Endogenous FLT1 signaling detected by positive staining for p-FLT1^{Y1333} (the kinase active domain) was mainly localized to gonocytes at P1, but heterogeneous low-grade expression was also detected in Sertoli cells and interstitial Leydig cells (Supplemental Fig. 2D). When compared with P1, the activation of FLT1 signaling was greatly increased in somatic cells based on increased staining intensity for p-FLT1^{Y1333} in Sertoli cells, Leydig cells, and lymphatic endothelial cells at P3. Interestingly, activated FLT1 signaling was restricted to a subset of pericentrally localized gonocytes in the seminiferous tubules and a few lymphatic endothelial and Leydig cells from P3–P6 (Supplemental Fig. 2E). In contrast, the expression of p-FLT1^{Y1333} diminished in germ cells after gonocyte migration to the basement membrane and subsequent conversion into undifferentiated spermatogonia during P6–P8. Activation of FLT1 in germ cells did not occur after P8 until approximately P20 when positive staining for p-FLT1^{Y1333} was detected in type-B spermatogonia and preleptotene primary spermatocytes (Supplemental Fig. 2F). Thus, FLT1 signaling appears to be related to survival and migration of gonocytes and other differentiated germ cell types.

In contrast to FLT1, detection of KDR signaling in the testis after birth was variable. At P1, very few gonocytes expressed p-KDR^{Y1054} (Supplemental Fig. 2G); however, this proportion increased by P3 (Supplemental Fig. 2H), the time period in which gonocytes resume proliferation. Sertoli cells from P1–P3 are positive for p-KDR^{Y1054}; however, by P5, Sertoli cells are negative, whereas undifferentiated spermatogonia are positive for p-KDR^{Y1054} (Supplemental Fig. 2H). Activation of KDR in Sertoli cells occurs around P6 and remains strong until P8, when spermatogonia lose the positive signal for p-KDR^{Y1054}.

NRP1 signaling appears to be minimal within the seminiferous tubules after birth based on low staining intensity for p-NRP1^{T916} in gonocytes and Sertoli cells (Supplemental Fig. 2, J and K). Immunohistochemical analysis demonstrated that NRP1 activation in gonocytes, Sertoli cells,

and undifferentiated spermatogonia occurs from P3–P8 (Supplemental Fig. 2K) and then declines until adulthood. NRP1 activation is rare by P14 and was observed in only approximately 5–6% of tubule cross-sections evaluated (one of 18), being localized and restricted to single undifferentiated spermatogonia in tubule cross-sections (Supplemental Fig. 2L).

VEGF isoforms regulate testis development and the biological activity of undifferentiated germ cells *in vivo*

After assaying the expression pattern of VEGF family molecules and evaluating the timeline for VEGFR activation during neonatal testis development, we sought to determine the role of specific VEGF isoforms in regulating SSC cell fate decisions. To accomplish this objective, we used the approach of treating neonatal (P3–P5) mice *in vivo* using two experimental paradigms (Fig. 3A). Briefly, mice received daily ip injections of 1) control (vehicle alone; 10 mM PBS plus 0.1% BSA), VEGFA₁₆₄ (500 ng, wt/vol), or VEGFA_{165b} (500 ng, wt/vol) and 2) IgG control (1 μg, wt/vol), anti-VEGFA₁₆₄ (1 μg, wt/vol), or anti-VEGFA_{165b} (1 μg, wt/vol). It is known that a decline in testis weight indicates impaired cellular development or disruption of spermatogenesis in the postnatal testis. Thus after killing, body weight and paired testes weights were recorded at P8 and P22. When compared with vehicle alone, no difference in testis weight was detected at P8 after VEGFA₁₆₄ or VEGFA_{165b} treatment (Fig. 3B). However, we observed a significant reduction ($P = 0.005$) in testis weight when killed at P22 in mice treated with VEGFA_{165b} from P3–P5 when compared with testis weights in control or VEGFA₁₆₄ treatment groups. Moreover, inhibiting the biological activity of VEGFA₁₆₄ with anti-VEGFA₁₆₄ significantly decreased ($P = 0.0242$) testis weight at P8, when compared with IgG control or the anti-VEGFA_{165b} treatment groups, respectively (Fig. 3C). No differences in testis weight were detected when mice were evaluated at P22 (Fig. 3C). Thus, VEGFA isoforms are likely important regulators of neonatal testis development *in vivo*.

We also evaluated our treatment approach on the kinetics of germ and Sertoli cell proliferation and survival in a subset of mice by counting these cells at each biological endpoint. Regardless of treatment, no differences in Sertoli cell number were detected at P8–P22 (data not shown). We assayed the number of germ cells in testes from treated and control animals to determine whether treatment altered the initiation or pace of germ cell differentiation. Immunohistochemistry with an antibody for the germ cell-specific marker DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4) was used to count germ cells. DDX4 is expressed by all germ cells in developing testes providing a useful marker to determine whether changes in the SSC or undifferentiated spermatogonia population lead to changes in meiotic germ cells. Treating mice with VEGFA_{165b} significantly increased the number of germ cells (DDX4⁺ cells) present at P8, but not P22, when compared with controls (Fig. 4A); a correlated increase in seminiferous tubule diameter was also observed (data not shown). Similarly, VEGFA_{165b} treatment significantly increased the number of spermatogonia localized at the basement membrane at P8 (Fig. 4C), whereas blocking VEGFA_{165b} activity with an antibody resulted in germ cell loss at P8 (Fig. 4D), suggesting a cytoprotective role for VEGFA_{165b}. Thus, a balance with respect to the actions of VEGFA isoforms may be required to support the proliferation, maturation, and survival of undifferentiated spermatogonia. Light micrographs demonstrating immunohistochemical analysis of DDX⁺ cells in P8 and P22 testis tissue after *in vivo* treatment are provided (Fig. 4, E and F).

After treatments, mice were killed at P8, P12, and P22 and evaluated for several factors associated with SSC self-renewal (*bcl6b* and *nanos2*) and early differentiation (*neurog3*) with the use of qRT-PCR. TaqMan PCR assays were used to determine the *in vivo* effect of VEGFA isoform treatments on the mRNA expression of *bcl6b*, *nanos2*, and *neurog3* in developing mouse testis tissue. Regardless of treatment, no significant differences were observed in *bcl6b*, *nanos2*, or *neurog3* expression at P8 or P12 (Fig. 5, A–F). However, VEGFA₁₆₄ and VEGFA_{165b} treatments resulted in a significant decrease of *bcl6b* and *neurog3* expression *in vivo* at P22 (Fig. 5A) compared with controls, whereas *nanos2* expression was not affected (Fig. 5C). Blocking the activity of VEGFA_{165b} isoforms ($P < 0.05$) increased *nanos2* and *neurog3* expression when compared with controls and anti-VEGFA₁₆₄ groups at P22, respectively (Fig. 5, D and F). These data indicate that a balance between VEGFA isoform signal transduction may function to regulate the cell fate decisions of SSC to either self-renew or differentiate.

VEGF isoforms regulate *in vivo* SSC homeostasis after germ cell transplantation analysis

Germ cell transplantation analysis was used to determine the effect of VEGFA isoform treatments from P3–P5 on SSC formation at P8 and subsequent proliferation of the adult SSC population at P22. To accomplish this goal, testis tissue from VEGFA isoform and antibody-treated Rosa26⁺ mice were obtained at P8 and P22 and digested to obtain a single-cell suspension of germ cells for transplantation into the testis of infertile, busulfan-treated recipient mice (Fig. 6). Eight weeks after transplantation, recipient mice were killed, testes collected and stained with 5-bromo-4-chloro-3-indo-1-yl β -D-galactoside, and the number of blue, donor-derived stem cell colonies was recorded (Fig. 6, A and B). In addition to calculating the number of SSC, we also assayed the postcolonization expansion rate of our donor-derived SSC to determine the effect of treatment on SSC self-renewal at P8 and maturation at P22, respectively (Fig. 6, C and D).

Germ cells obtained from VEGFA₁₆₄-treated mice contained the same number of SSC as controls at P8 and P22, respectively (Fig. 7A). In contrast, *in vivo* VEGFA_{165b} treatment significantly decreased the colonization efficiency of SSC, as evidenced by a 2-fold or greater reduction in donor-derived colonies detected at P8 and P22 ($P \leq 0.05$), when compared with control and VEGFA₁₆₄ treatments (Fig. 7A). Interestingly, donor germ cells harvested at P8 from mice treated with antibodies against VEGFA₁₆₄ and VEGFA_{165b} contained significantly less SSC than controls after 8 wk transplantation (Fig. 7B). However, a compensatory increase in the number of SSC obtained from mice treated with anti-VEGFA₁₆₄ was detected by P22, based on the number of donor-derived colonies observed after transplantation (Fig. 7B). In contrast, the number of SSC in donor mice injected with anti-VEGFA_{165b} remained significantly lower ($P \leq 0.05$) than control and anti-VEGFA₁₆₄-treated mice, respectively (Fig. 7B). These data indicate that VEGFA₁₆₄ may be critical for SSC formation and self-renewal, whereas VEGFA_{165b} likely regulates SSC differentiation and/or survival.

Analysis of colony expansion demonstrated that VEGFA_{165b} treatment decreased, whereas blocking VEGFA_{165b} significantly increased the growth and expansion rate of P8 donor SSC when compared with controls and anti-VEGFA₁₆₄ groups (Fig. 7, C and D). Conversely, donor SSC harvested from P22 mice treated with VEGFA₁₆₄ demonstrated reduced colony growth

following transplantation when compared with vehicle alone (Fig. 7C). Interestingly, blocking the activity of VEGFA_{165b} produced similar effects in P22 donor SSC, evidenced by a 3-fold decrease in colony growth and expansion in reference to controls (Fig. 7D). This disparity suggests that a balancing act between VEGFA isoform signaling functions to regulate the cell-fate decisions of SSC *in vivo*. The results of transplantation indicate diverse roles for VEGFA isoform activity with respect to regulating SSC homeostasis *in vivo*.

Discussion

VEGFA, known for its role as a potent endothelial cell mitogen (22), has been implicated in a variety of nonvascular processes including neurogenesis (39–44), myogenesis (45–47), granulosa cell function (48–50), and testis development (15, 17, 51, 52). In humans, the *VEGFA* gene spans 16,272 bp of chromosome 6p12 and consists of eight exons (19). Alternative splicing of VEGFA yields proangiogenic isoforms (VEGFA₁₁₁, VEGFA₁₂₁, VEGFA₁₄₅, VEGFA₁₆₂, VEGFA₁₆₅, VEGFA₁₈₃, or VEGFA₂₀₆) and antiangiogenic isoforms (VEGFA_{121b}, VEGFA_{145b}, VEGFA_{165b}, VEGFA_{183b}, or VEGFA_{189b}) in regard to vascular development (19), but the role of these isoforms in nonvascular processes have yet to be elucidated. Accordingly, the complexity of VEGFA signal transduction *in vivo* increases because each of the 14 known VEGFA splice variants vary slightly in terms of biological activity, mechanism of receptor activation, and binding affinity for receptor/coreceptor interactions despite overlapping expression within a given cell type (19, 22, 32, 53–58).

Recent studies have found that SSC self-renewal, proliferation, and differentiation depend on the contributions of extrinsic and intrinsic factors modulated through the somatic cell niche (8). Identification of the network of factors regulating the biological activity of SSC is difficult due to the lack of specific SSC markers. As a result, *in vivo* treatment and transplantation studies are critical for a better understanding of the underlying mechanisms directing SSC cell fate.

In this study, we used qRT-PCR and immunohistochemical analysis to demonstrate the age- and cell-specific expression of the major VEGFA family ligands and receptors that coincide with specific events critical for germ cell development. The pattern of ligand expression we observed supports the hypothesis that autocrine and paracrine VEGFA production by germ and Sertoli cells is important for regulating SSC proliferation, self-renewal, and differentiation. Just as importantly, these ligands elicit their effects after binding to receptors FLT1 and KDR and the cofactor NRP1 in endothelial (22, 24, 57, 59–63) and nonvascular (37, 41, 44, 46, 49, 64–68) cell types. We demonstrate here differential expression of these receptors in gonocytes, undifferentiated spermatogonia, and Sertoli cells within the seminiferous epithelium during postnatal development. Due to the complex cell biology of the testis and activity of VEGFA isoforms, we also characterized the cell types affected by VEGFR signaling *in situ*, using phosphorylation-specific antibodies against the major intracellular kinase-active domains of FLT1, KDR, and NRP1. We observed robust FLT1 signal transduction in mitotically inactive and migrating gonocytes shortly after birth, implicating its inherent role in regulating gonocyte survival, proliferation, and migration. In contrast, strong KDR and NRP1 activation was restricted to proliferating gonocytes and undifferentiated spermatogonia present on the basement membrane from P3–P8, and NRP1 activation appears to be restricted to a small population of undifferentiated spermatogonia from P14 to adulthood. The Sertoli cell pattern of FLT1, KDR,

and NRP1 activation correlates well with the differential expression of VEGFA ligands observed and, together with our germ cell data, support the hypothesis that VEGFA isoforms regulate SSC either directly or in concert with somatic niche cells.

Thus, we hypothesized that VEGFA family isoforms play an important role in regulating the cell fate decisions of SSC and used functional transplantation to test the effects of VEGFA₁₆₄ or VEGFA_{165b}, the two most potent pro- and antiangiogenic variants, on SSC self-renewal and differentiation *in vivo*. Based on colony number and growth rate after ligand and antibody treatment, we conclude that proangiogenic VEGFA₁₆₄ supports SSC proliferation and self-renewal in mice, extending our findings in bulls (17). In contrast, when donor mice are treated with VEGFA_{165b}, their SSC have a reduced capacity for proliferation and self-renewal. Similarly, treatment with VEGFA_{165b} results in a larger number of spermatogonia present, demonstrating that VEGFA_{165b} has a role in stimulating SSC differentiation to produce spermatogonia. Blocking VEGFA_{165b} activity in mice resulted in a peculiar phenotype characterized by substantial loss of undifferentiated germ cells *in situ* and a smaller adult SSC population compared with controls. Thus, VEGFA_{165b} signaling may also elicit a cytoprotective response in germ cells, similar to findings in the retinal epithelial cells (69). These data demonstrate VEGFA isoform regulation of SSC homeostasis likely requires a balancing act between proangiogenic and antiangiogenic variants. We recognize the potential for VEGFA treatments to elicit systemic effects on gonadotropin levels, such as FSH, that may modulate somatic cell populations. However, this is unlikely because no difference in Sertoli cell number was observed at any time points in the present study. Alteration of VEGFA isoform production from angiogenic to antiangiogenic isoforms could shift SSC from self-renewal to differentiation. These findings may have implications associated with accelerated loss of sperm production due to loss of SSC or precocious SSC differentiation. Investigation of cell-specific mechanisms that regulate alternative splicing in the testis and the specific mechanisms by which VEGFA treatment affects SSC fate decisions will be the focus of future *in vitro* studies.

Several studies have demonstrated the importance of GDNF for maintaining SSC survival and self-renewal (9, 10, 70) via activation of ret proto-oncogene (RET)^{Y1062} signaling (71). This is intriguing because GDNF expression appears to be weak in the postnatal testis *in vivo* based on publicly accessible microarray datasets (72) and is expressed in both germ and somatic cells in the seminiferous tubule (8). Interestingly, VEGF signaling has been shown to cross talk with the GDNF pathway in the developing kidney by inducing RET^{Y1062} phosphorylation and up-regulation of GDNF expression (73) in a pattern suggesting a positive feedback loop exists for these growth factors. These observations, along with the pattern of VEGFA isoform and receptor expression by germ and somatic niche cells, suggest an intricate, multifactor network supporting the maintenance of SSC and the initiation of spermatogonia differentiation. To our knowledge, this is the first report of active VEGFR signaling detected in male germ cells, and more importantly, functional transplantation of SSC demonstrates that VEGFA isoforms regulate the cell fate decisions of SSC *in vivo*. The functional roles VEGFA splice variants and the complex partnerships between receptors leading to regulating the biological activity of SSC will be the focus of future experimentation.

Supplementary Material

Supplemental Data:

Acknowledgments

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Footnotes

Abbreviations:

DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
FLT1	fms-related tyrosine kinase 1
GDNF	glial cell line-derived neurotrophic factor
KDR	kinase insert domain receptor
NRP1	neuropilin 1
P1.5	postnatal d 1.5
qRT-PCR	quantitative real-time RT-PCR
RET	ret proto-oncogene
SSC	spermatogonial stem cells
VEGFA	vascular endothelial growth factor A
VEGFR	VEGFA receptor.

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Figures and Tables

Table 1.

TaqMan PCR probes used for quantitative real-time RT-PCR assays

Assay ID	Gene symbol	Gene name
Mm00455914_m1	<i>Bcl6b</i>	B-cell CLL/lymphoma 6 member B protein
Mm01210866_m1	<i>Flt1</i>	Fms-related tyrosine kinase 1
Mm00599849_m1	<i>Gdnf</i>	Glial cell line-derived neurotrophic factor

Assay ID	Gene symbol	Gene name
Mm00833897_m1	<i>Gfra1</i>	Glial cell line-derived neurotrophic factor family receptor α 1
Mm01222419_m1	<i>Kdr</i>	Kinase insert domain receptor
Mm02525720_s1	<i>Nanos2</i>	Nanos homolog 2
Mm00437606_s1	<i>Neurog3</i>	Neurogenin 3
Mm00435379_m1	<i>Nrp1</i>	Neuropilin 1
Mm00803099_m1	<i>Nrp2</i>	Neuropilin 2
Mm00475529_m1	<i>Rps2</i>	Mitochondrial ribosomal protein S2
Mm00437304_m1	<i>Vegfa</i>	VEGFA

Table 2.

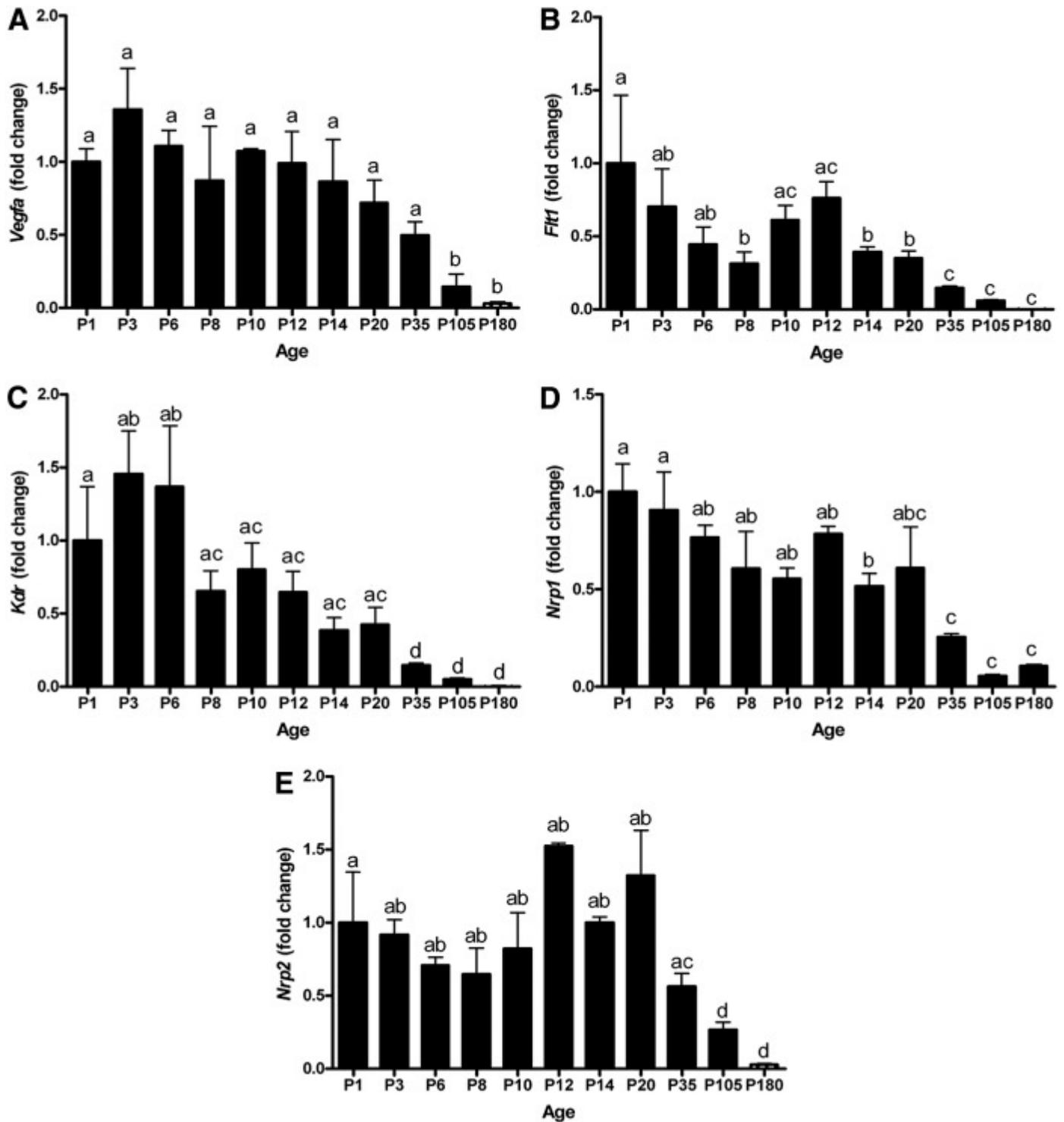
List of antibodies used for immunohistochemical analysis

Antigen	Antigen description	Dilution	Source
DDX4	Rabbit polyclonal to human DDX4/MVH	1:500	Abcam ^a
FLT1	Rabbit polyclonal to mouse FLT1 (C-17)	1:400	SCBT ^b
KDR	Mouse monoclonal to human KDR (A-3)	1:400	SCBT ^b
NRP1	Rabbit monoclonal to human NRP1 (EPR3113)	1:200	Abcam ^a
NRP2	Rabbit monoclonal to human NRP2 (ab39067)	1:200	Abcam ^a
VEGFA ₁₆₄	Rabbit polyclonal to mouse VEGFA (A-20)	1:200	SCBT ^b
VEGFA _{165b}	Mouse monoclonal to human VEGF 165b (MRVL56/1)	1:200	Abcam ^a

^aAbcam, Inc., Cambridge, MA.

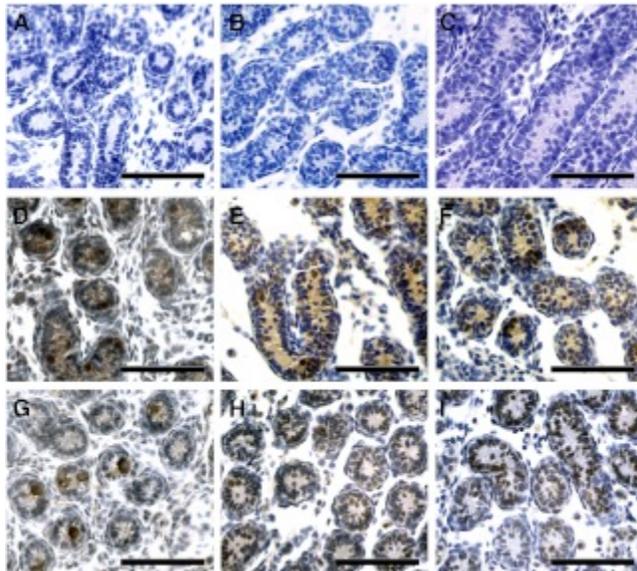
^bSanta Cruz Biotechnology, Santa Cruz, CA.

Fig. 1.



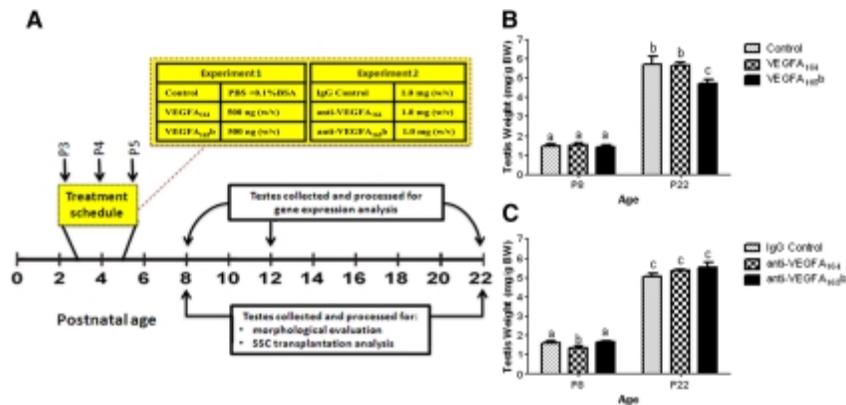
Expression of VEGFA family isoforms, receptors, and coreceptors in mouse testes. Quantitative measurement (real-time RT-PCR) of mRNA of *Vegfa* (A), *Flt1* (B), *Kdr* (C), *Nrp1* (D) and *Nrp2* (E) from birth to adulthood. Data are representative of three independent experiments (n = 3), and different letters indicate significant differences between means ($P < 0.05$).

Fig. 2.



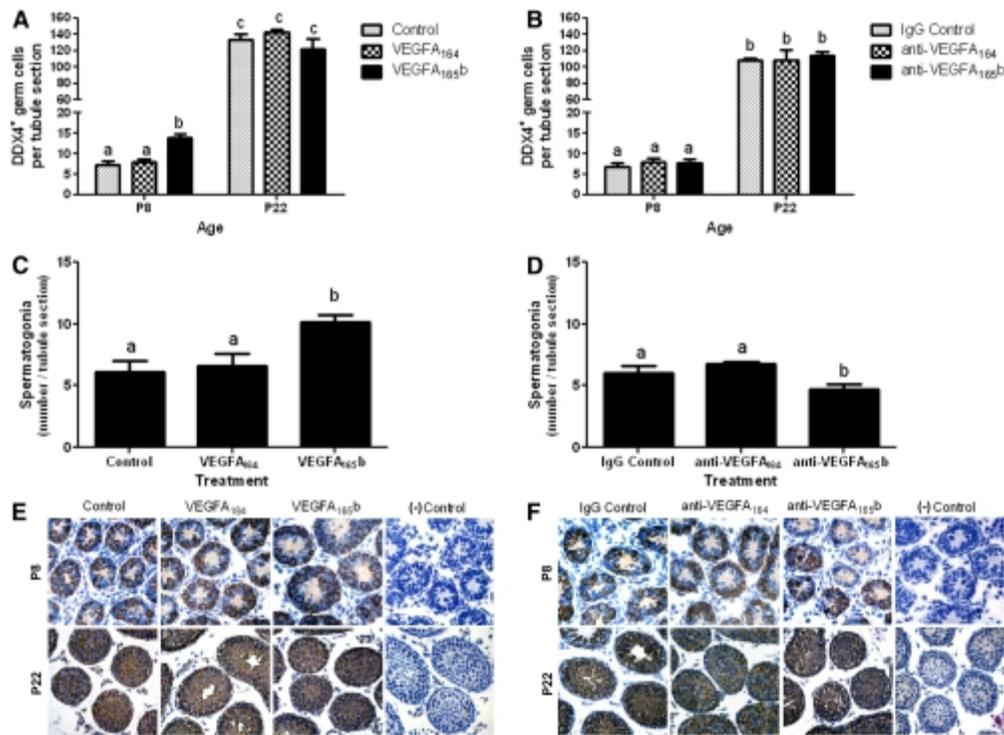
VEGFA isoforms are expressed by germ and somatic cells during testis development. Immunohistochemical analysis of VEGFA₁₆₄ (D–F) and VEGFA_{165b} (G–I), protein expression in the testis tissue of P3, P5, and P8 mice. Omitting primary antibody served as a negative control in testis tissue (A–C). Four donors were used for each age and antigen evaluated. *Scale bars, 50 μm.*

Fig. 3.



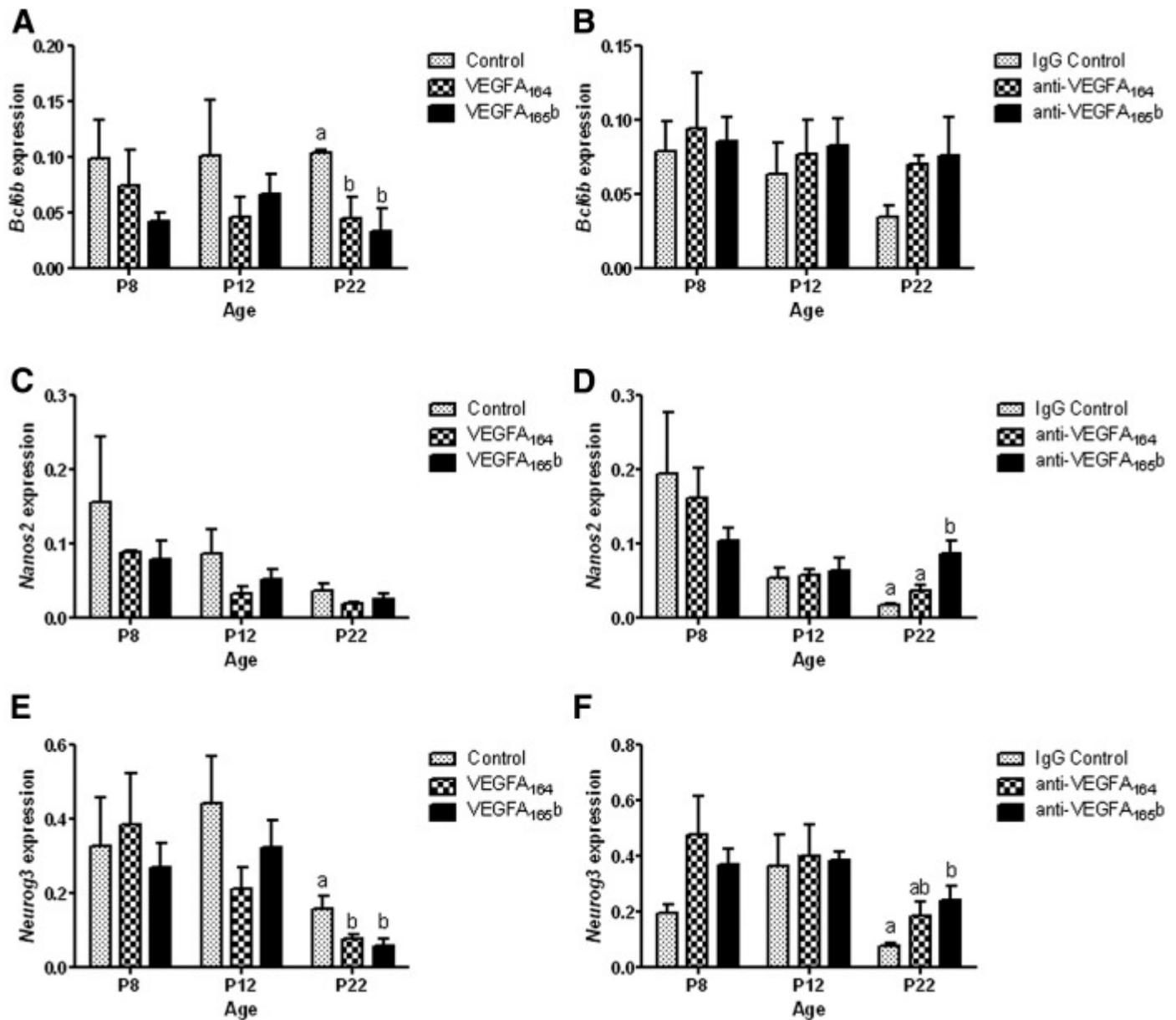
VEGFA isoforms regulate mouse testis development *in vivo*. A, Diagram of the treatment schedule for experiments; B, testis weights of mice after VEGFA₁₆₄, VEGFA_{165b}, and control treatments; C, testis weights of mice after antibody treatments to block the biological activity of VEGFA₁₆₄ and VEGFA_{165b} in addition to treatment with nonspecific IgG as a control. Data are representative of at least three independent experiments ($n \geq 3$), and different *letters* indicate significant differences between means ($P < 0.05$).

Fig. 4.



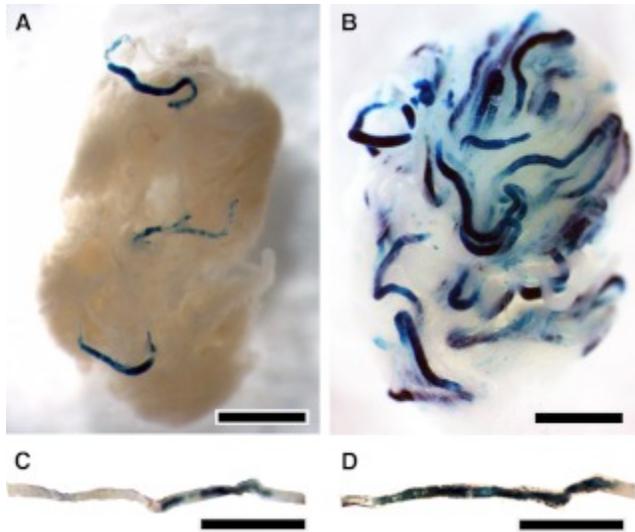
VEGFA isoforms regulate the biological activity of undifferentiated germ cells *in vivo*. Effect of VEGFA isoform ligand (A) and antibody (B) treatments daily from P3–P5 on the number of germ cells present in the testes of P8 and P22 mice. VEGFA isoform ligand (C) and antibody (D) treatments daily from P3–P5 on spermatogonial numbers present in the testes of P8. Germ cells were identified and counted with the use of immunohistochemical localization of DDX4, a germ cell-specific marker. E and F, Representative images of DDX4-stained testis tissue in P8 (E) and P22 (F) mice, respectively. Data are representative of three independent experiments ($n = 3$), and different *letters* indicate significant differences between means ($P < 0.05$). *Scale bars*, 50 μm .

Fig. 5.



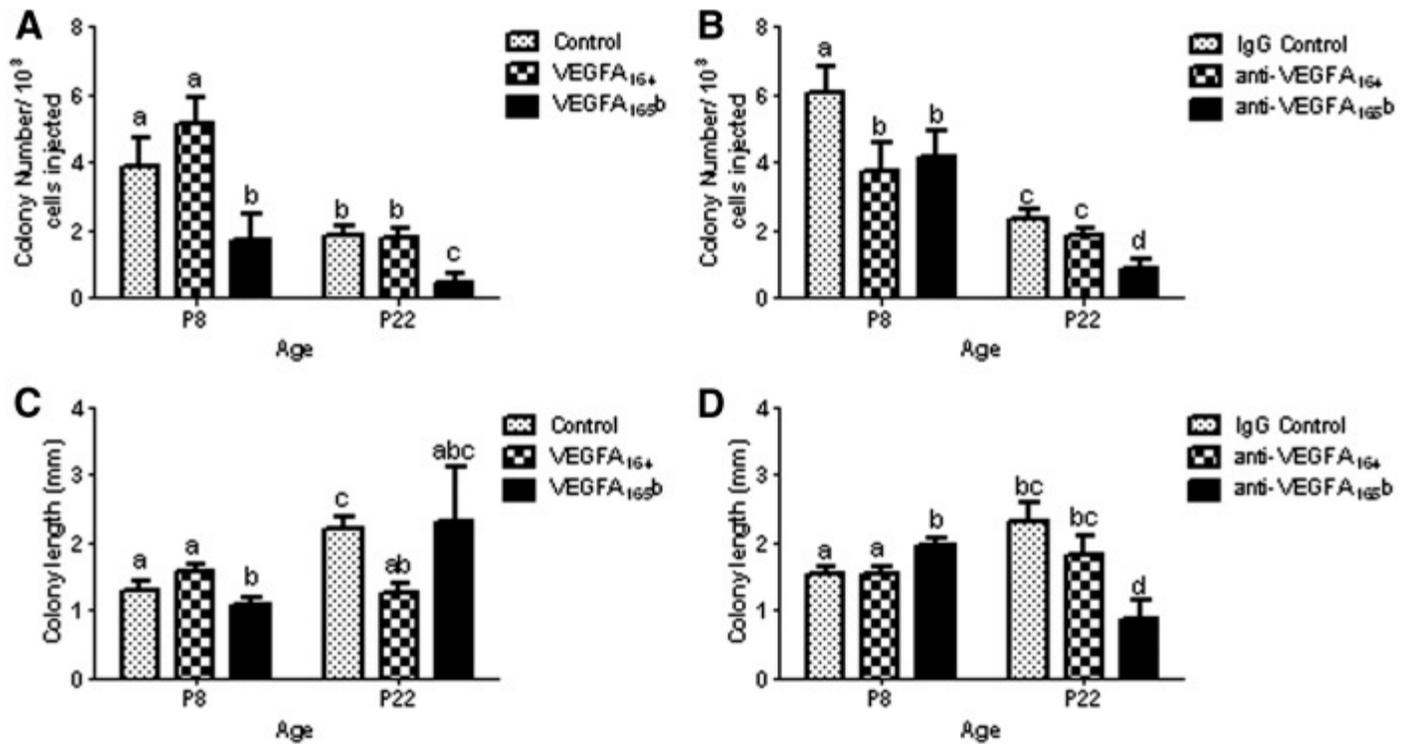
Effect of VEGFA isoform ligand (A, C, and E) and antibody (B, D, and F) treatments daily from P3–P5 on the expression of SSC niche-associated factors in P8, P12, and P22 mice, respectively. A–F, The normalized, mean expression values of *Bcl6b* (A and B), *Nanos2* (C and D), and *Neurog3* (E and F). Data are representative of at least three independent experiments, and different letters indicate significant differences between means ($P < 0.05$).

Fig. 6.



Representative images of recipient testes 8 wk after cell transplantation used to assay the effects of VEGFA ligand and antibody treatments daily from P3–P5 on regulating the biological activity of donor SSC. Light micrographs of recipient testes 8 wk after transplantation with SSC colony formation as indicated by areas of blue staining. A, Representative recipient testis with SSC colonization and donor-derived spermatogenesis after transplantation of germ cells at P8 after VEGFA_{165b} treatment from P3–P5 in the donor mouse. Note the limited number of blue areas representing SSC colonization; B, representative recipient testis with SSC colonization after transplantation of germ cells at P8 after VEGFA₁₆₄ treatment from P3–P5 in the donor mouse; C, a representative seminiferous tubule from a recipient testis with low rate of SSC expansion such as observed in the recipient testes transplanted with donor germ cells at P8 after VEGFA_{165b} treatment; D, a representative seminiferous tubule from a recipient testis with a high rate of SSC expansion such as observed in the recipient testes transplanted with germ cells from P8 donor mice treated with the anti-VEGFA_{165b} antibody. Data are representative of at least four independent experiments ($n \geq 4$), and different *letters* indicate significant differences between means ($P < 0.05$). *Scale bars*, 0.5 mm (all *panels*).

Fig. 7.



Effect of VEGFA family isoforms on colonization efficiency of donor-derived SSC originating from mice treated from P3–P5 and transplanted at either P8 or P22. Recipient testes were analyzed 8 wk after transplantation. A, SSC colony number in testes of recipient mice after VEGFA₁₆₄, VEGFA_{165b}, or control treatment; B, SSC colony number in testes of recipient mice after anti-VEGFA₁₆₄ antibody, anti-VEGFA_{165b} antibody treatment, or treatment with nonspecific IgG control; C and D, SSC colony growth and expansion after VEGFA₁₆₄, VEGFA_{165b}, or control treatment (C) and SSC colony growth and expansion after anti-VEGFA₁₆₄ antibody, anti-VEGFA_{165b} antibody treatment, or treatment with nonspecific IgG control (D). Data are representative of at least three independent experiments, and different *letters* indicate significant differences between means ($P < 0.05$).