The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells

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We recently identified an angiogenic mitogen, endocrine-gland-derived vascular endothelial growth factor (EG-VEGF), with selective activity for endothelial cells of endocrine tissues. Here we describe the characterization of a highly related molecule, Bv8, also known as prokineticin-2. Human Bv8 shares 60% identity and 75% similarity with EG-VEGF. The human and mouse Bv8 genes share a common structure. Like EG-VEGF, Bv8 is able to induce proliferation, survival and migration of adrenal cortical capillary endothelial cells. Bv8 gene expression is induced by hypoxic stress. Bv8 expression occurs predominantly in the testis and is largely restricted to primary spermatocytes. Adenoviral delivery of Bv8 or EG-VEGF to the mouse testis resulted in a potent angiogenic response. We have localized the expression of the Bv8/EG-VEGF receptors within the testis to vascular endothelial cells. The testis exhibits relatively high turnover of endothelial cells. Therefore, Bv8 and EG-VEGF, along with other factors such as VEGF-A, may maintain the integrity and also regulate proliferation of the blood vessels in the testis.

mitogen | survival | migration | G protein-coupled receptor | prokineticin

The embryonic vascular system evolves from the primary capillary plexus by pruning and reorganization of endothelial cells in a process termed angiogenesis (1). Angiogenesis is also essential in adult physiology for wound healing and, prominently, for reproductive functions (2). Although angiogenesis is most commonly associated with the cyclical proliferative events in the female reproductive tract, most species also display a surprisingly high rate of endothelial cell turnover within the testis (3).

The diverse structural and functional properties of endothelial cells, and evidence from a variety of *in vivo* (4–7) and *ex vivo* systems (8, 9), suggest the existence of local, tissue-specific regulators of endothelial cell phenotype and growth. Recently, we reported the identification of a tissue-specific angiogenic factor, endocrine-gland-derived vascular endothelial growth factor (EG-VEGF) (10). Expression of human (h)EG-VEGF mRNA is principally restricted to the steroidogenic glands: ovary, testis, adrenal, and placenta. EG-VEGF promoted proliferation, migration, and fenestration in cultured adrenal capillary endothelial cells. It also induced extensive angiogenesis when delivered to the ovary, but not other tissues (10).

EG-VEGF is a member of a structurally related class of peptides including the digestive enzyme colipase, the *Xenopus* head-organizer, Dickkopf (11), venom protein A (VPRA) (12) or MIT-1 (13), a nontoxic component of *Dendroaspis polylepis polylepis* venom, and the secreted protein from *Bombina variegata*, designated Bv8 (14). The distinguishing structural motif is 10 cysteine residues that form five disulfide bridges within a conserved span, designated a colipase-fold (15). EG-VEGF (80% similar to VPRA) and VPRA are most closely related (83% and 79% similarity, respectively) to the Bv8 peptide. Mouse and human orthologues of Bv8, also known as prokineticin-2 (16), have been recently described, and a variety of activities for these proteins have been reported, including effects on neuronal survival (17), gastrointestinal smooth muscle contraction (16), and circadian locomotor rhythm (18).

In the present study, we characterized the primary structures of mouse and human (m- and hBv8) isoforms, the distribution of Bv8 expression, and its activity in the testis, the major site of Bv8 expression. Bv8 expression is mainly restricted to the primary spermatocytes. Like EG-VEGF, Bv8 functions as a mitogen, chemoattractant, and survival factor in cultures of adrenal cortical capillary endothelial (ACE) cells and is induced by hypoxic stress. Adenovirus-based delivery of Bv8 or EG-VEGF to the mouse testis resulted in a profound angiogenic response. Finally, we determined that endothelial cells within the testis interstitial tissue express two G protein-coupled receptors (GPCRs) for Bv8/EG-VEGF (19, 20).

Materials and Methods

Isolation of m- and hBv8 cDNAs and Genes. mBv8 cDNA was cloned from an embryonic day (E)15–16 library by using hEG-VEGF cDNA as probe. The human isoforms were cloned from a mixed library of 50 pools by using the mBv8 cDNA as probe. The sequence of the human gene is available (GenBank accession no. AC096970). The mouse gene was identified on a bacterial artificial chromosome (BAC) clone (Research Genetics, Huntsville, AL), by using a primer pair that amplified 172–297. The gene was subcloned as *Eco*RI fragments and sequenced by transposon insertion. Human and mouse sequences thus obtained matched those in a private database (Celera Discovery System, www.celera.com).

Isolation of Human and Mouse (m)EG-VEGF/Bv8 Receptors. Biochemical evidence indicated that the EG-VEGF receptor was a GPCR (21). Human cDNAs encoding the two cognate receptors for EG-VEGF and Bv8 were isolated by a bioinformatics approach that searched for candidate GPCR with a defined expression pattern. The candidates were cloned and assessed in a functional screen. Human receptor (R)-1 and -2 were cloned from testis and fetal brain libraries, respectively. The mouse orthologues were isolated from testis and spinal cord libraries, respectively. These sequences were recently reported (19, 20) and GenBank accession numbers are as follows: hR-1/ZAQ, AY089976; hR-2/I5E, AF506288; mR-1, NM138964; and mR-2, AF487279.

Expression Analyses. Tissue RNA arrays and blots were purchased from CLONTECH. cDNA probes were prepared using 50 ng of human or mouse coding sequence with methods described (10). Tissues were processed for *in situ* hybridization (ISH) (22) and [^{33}P]UTP-labeled RNA probes were generated as described

Abbreviations: VEGF, vascular endothelial growth factor; EG-VEGF, endocrine-gland-derived VEGF; hEG-VEGF, human EG-VEGF; mEG-VEGF, mouse EG-VEGF; VEGFR-2, VEGF receptor 2; AvBv8, adenovirus-delivered Bv8; mBv8, mouse Bv8; hBv8, human Bv8; ACE, adrenal cortical capillary endothelial; GPCR, G protein-coupled receptor; ISH, *in situ* hybridization; R-1 and -2, receptor 1 and 2; E*n*, embryonic day *n*.

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(23). Sense and antisense probes for hBv8 were synthesized from a cDNA fragment corresponding to nucleotides 533-1132; mBv8, nucleotides 886-1611; and mR-1, nucleotides 220–946. This probe is 81.2% identical to mR-2. TaqMan analysis was carried out as reported (10). Primers and probes are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Hypoxic Regulation Studies. RNA isolates from replicate, matched samples of 18- to 24-h normoxic (22% O_2) versus hypoxia-exposed (2% O_2) P19 mouse embryonic carcinoma cells (American Type Culture Collection) were prepared, and TaqMan analysis with 100 ng of total RNA was conducted in triplicate samples as described (10).

Expression and Purification of Recombinant Proteins. The coding sequence of $mBv8_{81}$ was subcloned into pBPH.His.c to generate a C-terminal GHHHHHHHH tag. This vector is derived from the baculovirus expression vector pVL1393 (PharMingen). Fusion protein was produced and assessed as described (10).

Cell Assays. Bovine ACE cells (passage 4–7) were routinely cultured in low-glucose DMEM (Life Technologies, Grand Island, NY) supplemented with 10% calf serum and 2 mM glutamine. Proliferation assays were performed as described (24). For survival assays, 3×10^5 ACE cells were cultured in six-well plates (Falcon) in complete medium for 24 h. Cells were then washed and cultured for 24 h in CS-C medium (Cell Systems, Kirkland, WA) plus or minus factors. Trypsinized cells were washed and incubated in Ca^{2+} assay buffer (10 mM Hepes, pH 7.4/140 mM NaCl/2.5 mM $CaCl_2$), with 1 μg/ml annexin-FITC (BioVision, Palo Alto, CA) and 1 μg/ml propidium iodide (PI; Molecular Probes). Cell staining was assessed by fluorescence-activated cell sorter (FACS) analysis. ACE cell migration assays were performed as reported (10).

Immunoblots. ACE cells were cultured in subconfluent conditions for 48 h, followed by incubation in serum-free DMEM supplemented with 0.1% BSA overnight. The medium was changed to DMEM supplemented with 0.1% BSA for 90 min before assay. Following stimulation, cells were directly lysed in 0.5–1 ml of buffer [50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton X-100/protease inhibitor mixture (Roche)/phosphatase inhibitors (Sigma)/1 mM Na_3VO_4] and immunoblotting for anti-phospho-ERK or anti-phospho-Akt was performed as described (21).

Adenovirus Production and Delivery. Adenovirus encoding LacZ, vascular endothelial growth factor (VEGF), and EG-VEGF were described (10). The cDNA encoding $Bv8_{81}$ was cloned into the cytomegalovirus (CMV) shuttle vector, and recombinant virus was produced according to the manufacturer's instructions (Strat-

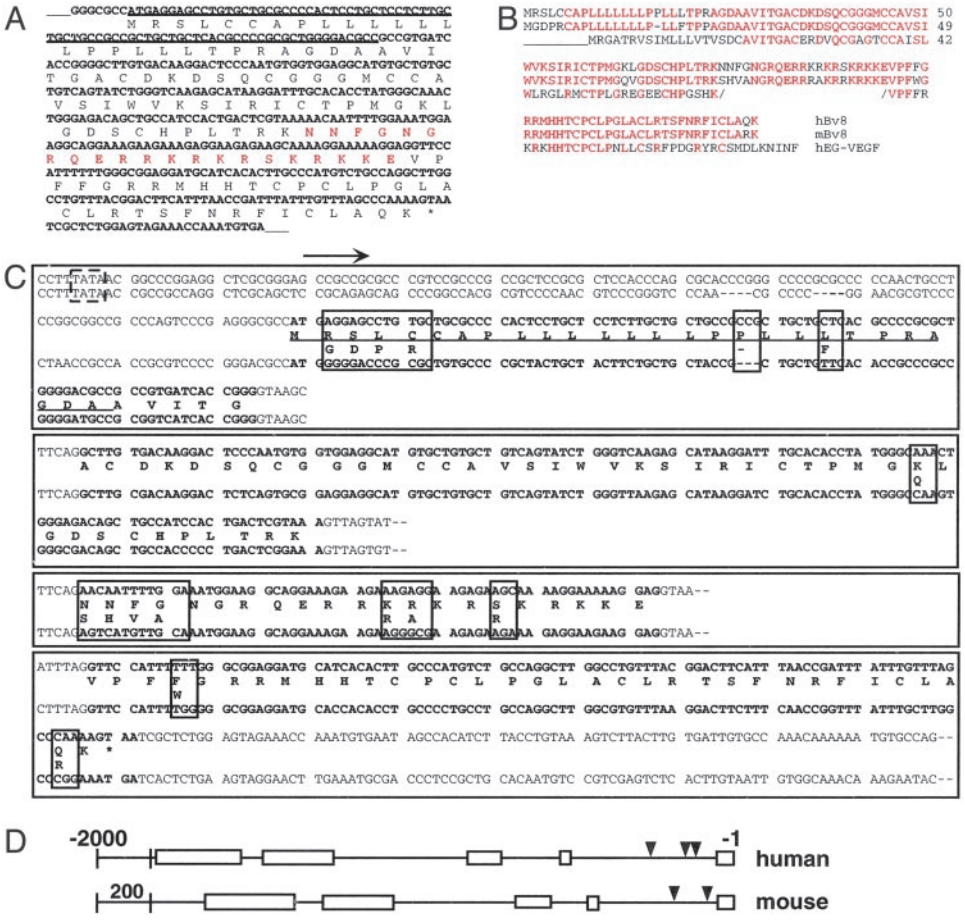

Fig. 1. Human and mouse primary sequences and gene structures. (*A*) hBv8 sequence. The putative transcription start is indicated by an arrow. The signal sequence is underlined and the residues in red are encoded in an alternative transcript. (*B*) The predicted amino acid sequences of hBv8, mBv8, and hEG-VEGF. Bv8 isoforms with a 21-aa insert are found in both human and mouse, whereas a corresponding longer form of EG-VEGF has not been identified. Identical amino acids are red. (*C*) Comparison of h- (upper sequence) and m*Bv8*. Exon 3 encodes the 21-aa insert of an alternate transcript. The coding sequence is in bold. (*D*) A comparison of ≈2,000 nt of human and mouse promoter regions revealed five distinct blocks of conservation (80–100% identity), representing 615 of 2,000 nt. Arrowheads represent putative hypoxia-response elements (HREs). Boxes are drawn to scale.

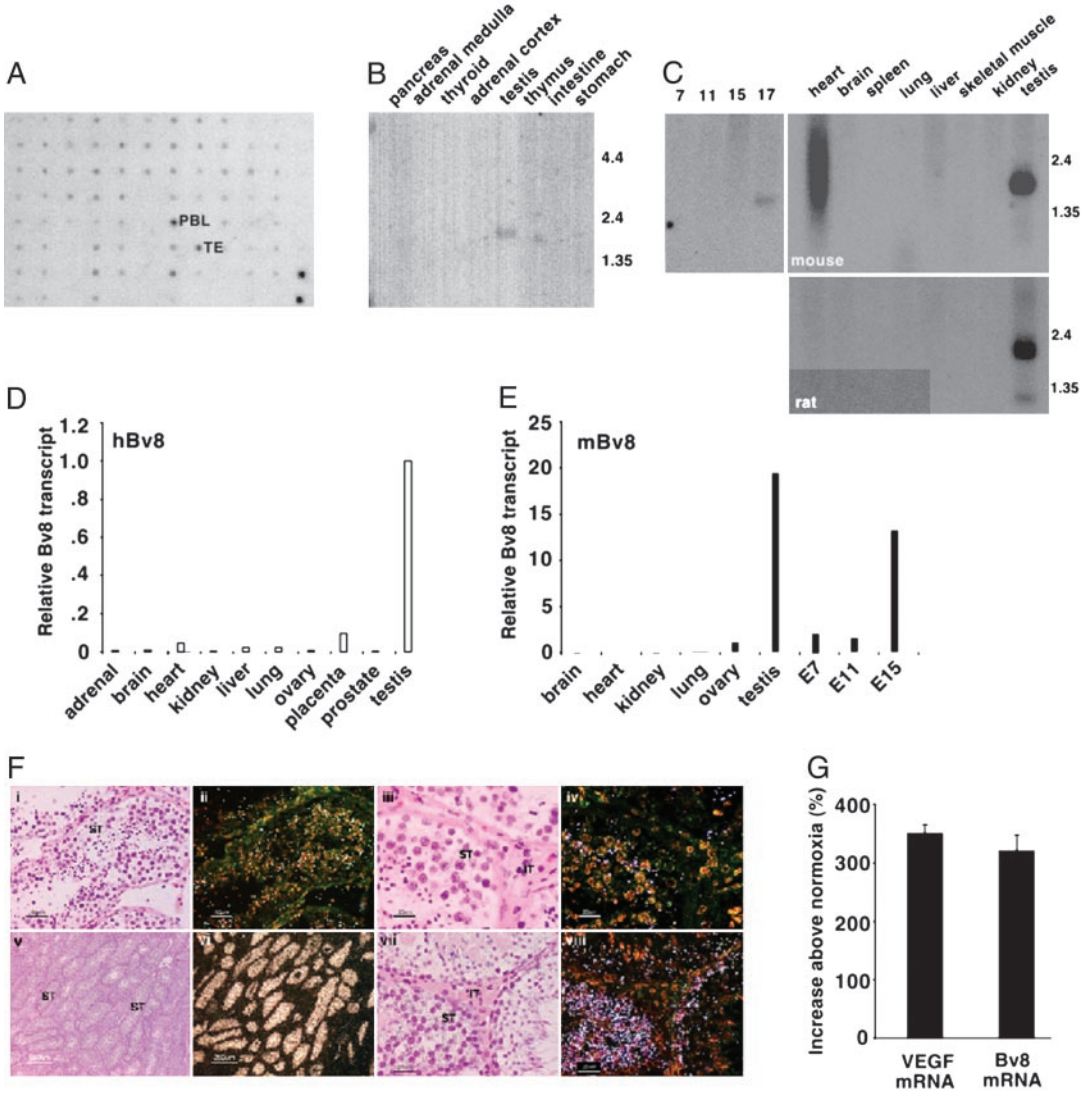

Fig. 2. Bv8 is predominantly expressed in the testis. (*A*) Hybridization of RNA dot blots revealed hBv8 signal in testis and peripheral blood leukocytes (PBLs). (*B*) By Northern blotting, a single 1.8-kb transcript was identified in testis. Equivalent loading was assessed with actin probe (not shown). (*C*) Bv8 mRNA is detected in late mouse embryogenesis, E17, and in testis, a transcript of 1.8 kb is most abundant. Samples and sizes (in kb) are indicated. TaqMan analysis of human (*D*) and mouse (*E*) samples revealed the highest Bv8 expression in the testis in either species, followed by the placenta in human, and by embryo and ovary in mouse. (*F*) ISH studies revealed restricted expression in adult human (*i–iv*) and mouse (*v–viii*) testis within primary spermatocytes. A developmental time course in mouse revealed expression at postnatal day 16, but not at postnatal day 7 or 10 (data not shown). Sense probes did not yield a signal. (*G*) TaqMan analysis of P19 cultures exposed to normoxic or hypoxic conditions revealed that the Bv8 transcript level increased 320 ± 27%, which is comparable to the 350 ± 15% increase in VEGF over normoxic controls. Data were normalized to GAPDH, and the value for normoxia was arbitrarily set at 100%. The graph represents three experiments. Error bars, SD.

agene). Virus was purified by using the kit from Virapur (Carlsbad, CA) and titered. Virus (5×10^8 pfu) was delivered in a volume of 10 μl by using a gas-tight Hamilton syringe. One week after unilateral injection into the testis, tissues were dissected, weighed, and fixed for histology. VEGF receptor 2 (VEGFR-2) immunostaining was performed as described (25). All studies were performed in accordance with institutional guidelines.

Results

hBv8 and mBv8 cDNAs and Gene Structures Are Highly Conserved. A low-stringency hybridization screen of a mouse embryo cDNA library identified Bv8 by using the hEG-VEGF cDNA as probe. This sequence was identical to that reported in refs. 26 and 27. Subsequently, a pool of 50 distinct human cDNA libraries was screened and two variants of the Bv8 orthologue were identified (Fig. 1 *A* and *B*). The gene structures of hBv8 (GenBank accession no. AC096970) and m*Bv8* are highly conserved (Fig. 1*C*). Exon 1 encodes the signal peptide and the first 5 aa of the mature protein. Exon 2 encodes 42 aa, including 6 of the 10 cysteines of the mature protein. Exon 3 encodes a 21-aa insert that is present in an alternative mRNA. Nineteen residues of this sequence are basic, reminiscent of the heparin-binding isoforms generated from the *VEGF* gene (28). The remaining 34 aa are encoded by exon 4, including 4 of the 10 cysteines of the secreted protein. The striking features of the predicted sequences include alternative splicing and the high cysteine content, potentially five disulfide bonds. We designate these possible variants $Bv8_{102}$ and $Bv8_{81}$. hBv8 and $mBv8_{102}$ share 82% identity and 87% similarity, respectively, and $Bv8_{81}$ isoforms share 95% identity and 99% similarity, respectively. hBv8 and hEG-VEGF are 60% identical and 75% similar (Fig. 1*B*). Presumably, these peptides adopt a colipase fold structure (29). The bioavailability of several se-

creted molecules, including basic fibroblast growth factor and VEGF, is regulated by interactions with extracellular matrix (ECM) components, such as heparin sulfate proteoglycans (30). We demonstrated that EG-VEGF binds with high affinity to heparin-Sepharose. Bv8 is also predicted to be highly basic, with calculated pI values of 8.85 for $Bv8_{81}$, and 10.68 for $Bv8_{102}$. Thus, Bv8 activity may also be regulated through binding to the ECM.

A direct comparison of 2,000 nt of mouse and human promoter sequences revealed five distinct blocks of high conservation (80–100% identity; Fig. 1*D*). These sequences account for greater than 30% (615/2,000 nt) of this promoter region.

Bv8 Is Highly Expressed in Human Primary Spermatocytes. To elucidate the expression pattern of hBv8, we performed dot-blot analysis on RNA arrays representing a panel of tissues and cell lines. Bv8 expression appeared restricted to testis and peripheral blood leukocytes (PBL; Fig. 2*A*). Northern blot analysis of human and mouse tissues verified these findings. A single mRNA species of 1.8 kb was detected in human (Fig. 2*B*), whereas two transcripts were detected in mouse and rat testis (Fig. 2*C*). With the exception of a weak signal in PBL, no expression was detected in other tissues examined (Fig. 2*B*, data not shown). These findings indicate that the testis is the major site of Bv8 expression in both human and rodents. To confirm this distribution, TaqMan analysis was performed (Fig. 2*D*). hBv8 mRNA is most prominently expressed in the testis, whereas placenta expresses ≈10% of this level. In the mouse, testis is also the tissue with highest expression, followed by the ovary (5% testis level) and the brain (0.35%). No specific mBv8 signal is detected in the heart, although a smear is present in the Northern blot. mBv8 expression appears to peak at E15–E17 of embryogenesis (Fig. 2 *C* and *E*). To identify the cell types that express hBv8, we examined a series of specimens by ISH. Within the seminiferous tubules of the testis, we detected a strong hybridization signal that was largely restricted to the primary spermatocytes (Fig. 2*F i–iv*). Whether a low-level expression occurs in secondary spermatocytes and spermatids remains to be established. These data are in agreement with published results in the mouse testis (26). No hybridization signal is detected before spermatocytic development (data not shown). At postnatal day (P)16, a signal is present within most seminiferous tubule cross-sections and localized to the most luminal (pachytene) spermatocytes (Fig. 2*F v–viii*).

Bv8 Expression Is Induced by Hypoxia. Hypoxia is an inducer of angiogenesis in both physiological and pathological conditions, and HIF-1α is a key mediator of the response (31). EG-VEGF expression is up-regulated under low oxygen conditions (10). Therefore, we sought to determine whether hypoxia regulates Bv8 expression. mBv8 is expressed in the P19 cell line under normal culture conditions. Exposure of these cultures to hypoxia resulted in a 320 ± 27% increase in Bv8 mRNA levels above normoxia, whereas the VEGF mRNA increased 370 ± 15% (Fig. 2*G*). A search of the m*Bv8* promoter sequence for the core HIF-1α binding site revealed two putative elements within the first 500 nt of the transcription start site, based on a consensus sequence (TA**CGTG**CGGC, invariable sequence in bold). These elements are located at −115 (CA**CGTG**CGCG) and −248 (CA**CGTG**AGGC). The human proximal promoter contains putative sites at −160 (CA**CGTG**CTCG), −201 (TG**CGTG**GCGC), and −340 (CA**CGTG**GAGC) (Fig. 1*D*). Additional putative hypoxia-response elements (HREs) are present in both promoters.

Bv8 Promotes Proliferation, Survival, and Migration of Endothelial Cells. Bv8 and EG-VEGF appear to share two receptors. Therefore, we sought to test whether Bv8, like EG-VEGF, could also act as an angiogenic mitogen. Bv8 induced a 2.0- to 2.5-fold increase in the ACE cell number, essentially identical to the EG-VEGF response (Fig. 3*A*). To further examine the functional overlap of these molecules, we tested whether Bv8 promoted survival of ACE. After

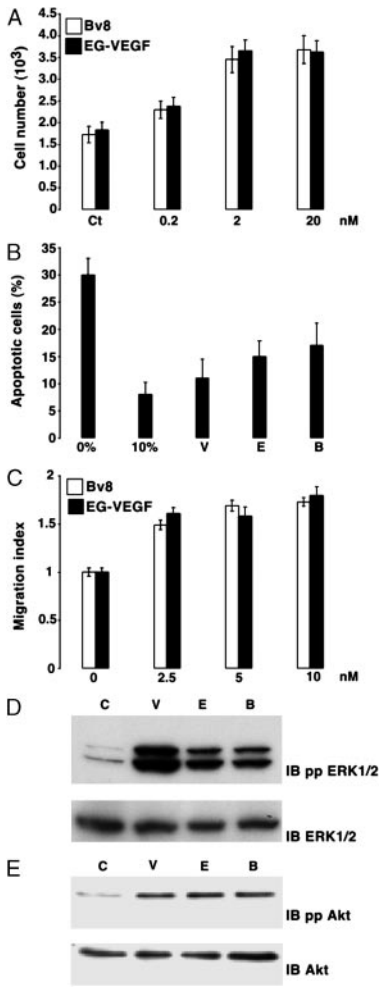

Fig. 3. Bv8 induces proliferation, chemotaxis, and survival of ACE cells. (*A*) Dose–response experiment comparing cell numbers in ACE cultures treated with Bv8 or EG-VEGF; negative control (Ct) was medium without factors. These molecules promote proliferation to a similar extent. (*B*) The percent apoptotic cells was scored after incubation in serum-free medium (0%), or with addition of 10% FCS, 2 nM (V)EGF, 20 nM (B)v8, or 20 nM (E)G-VEGF. Bv8 or EG-VEGF induced equivalent responses. (*C*) Bv8 and EG-VEGF induced migration of ACE cells to a similar extent. Error bars, SD. (*D*) ERK1/2 phosphorylation was assessed by immunoblot (IB) of ACE lysates, unstimulated (C), or treated with VEGF, Bv8, or EG-VEGF. Total ERK1/2 protein level is presented in *Lower*. (*E*) After a 15-min stimulation, VEGF, Bv8, and EG-VEGF increased phosphorylated Akt levels. Total Akt was assessed in *Lower*. In *A–E* representative experiments are shown.

24 h, cultures in complete media contained 8 ± 2.2% apoptotic cells, serum-free media conditions resulted in 30 ± 3.0%, 20 nM Bv8 reduced this value to 17 ± 4.1%, 20 nM EG-VEGF 15 ± 2.8%, and VEGF 11 ± 3.5% (Fig. 3*B*). Thus Bv8, like EG-VEGF and VEGF, is an endothelial cell survival factor. EG-VEGF promoted migration of select endothelial cells (10). Therefore, we compared EG-VEGF and Bv8 activities (Fig. 3*C*). Both molecules induced a significant 1.5- to 1.8-fold increase in migrated ACE cells, indicating they may function as endothelial cell chemoattractants *in vivo*.

Recently, the signal transduction events initiated by EG-VEGF in ACE cultures were reported (21). Given that Bv8 promotes mitogenesis and survival of cells, ERK and Akt activation were evaluated by using phosphorylation-specific antibodies. Comparable to EG-VEGF, Bv8 markedly induced phosphorylation of ERK1/2 within 10 min of stimulation (Fig. 3*D*). Akt phosphorylation was markedly increased above control cultures at 15 min of incubation with Bv8, EG-VEGF, or VEGF (Fig. 3*E*).

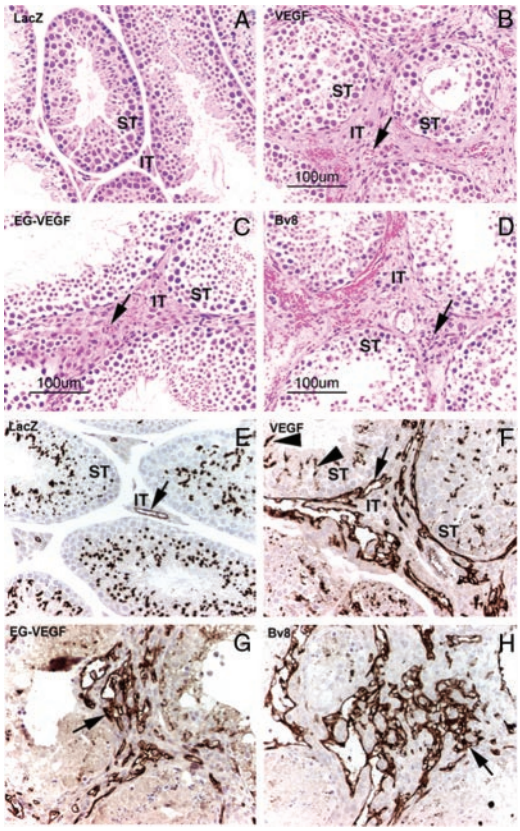

Fig. 4. AvBv8, EG-VEGF, or VEGF induces a potent angiogenic response in the testis. (*A*) Mouse testes injected with AvLacZ (or PBS; data not shown) demonstrated normal architecture, weight, and overall appearance. (*B–D*) AvVEGF (*B*), AvEG-VEGF (*C*), or AvBv8 (*D*) increased interstitial capillaries. Evidence of tubular atrophy in many samples suggested an increased interstitial pressure, presumably secondary to the angiogenic response. Note the high content of extravasated product within the interstitium of *B–D*. Immunohistochemistry for VEGFR-2 shows increased capillary densities in testes injected with AvVEGF (*F*), AvEG-VEGF (*G*), and AvBv8 (*H*). Few capillaries are apparent in the interstitium of control (*E*). Interestingly, early spermatids are also positive for VEGFR-2. Expression of VEGFR-1 by these cells was reported (40). Arrows indicate endothelium and arrowheads indicate early spermatids. ST, seminiferous tubule; IT, interstitial tissue.

Adenovirus-Based Delivery of Bv8 Induces Angiogenesis in the Testis. We tested whether adenovirus-delivered (Av)Bv8 may induce angiogenesis in testes. AvBv8-, EG-VEGF-, or VEGF-treated tissues exhibited focal hemorrhage by gross observation and respectively weighed 138 ± 7%, 140 ± 12%, and 165 ± 15.6% of the contralateral tissue. The contralateral tissue from each of these groups was indistinguishable from sham- or AvLacZ-injected tissues. Histopathological analysis revealed the presence of increased interstitial capillaries in the Bv8, EG-VEGF, and VEGF groups, and localized hemorrhage consistent with erythrocyte extravasation from leaky vessels (Fig. 4). Increased capillary density was verified by immunohistochemistry for VEGFR-2 (Fig. 4 *F–H*). A number of animals in the Bv8, EG-VEGF, and VEGF groups had evidence of tubular atrophy, possibly secondary to the increased interstitial pressure resulting from the angiogenic response. At the 1-week time point, the tissues injected with AvBv8, EG-VEGF, or VEGF were indistinguishable. No angiogenesis was observed in the PBS or AvLacZ groups.

Endothelial Cell-Restricted Expression of Bv8 Receptor(s) in Testis. The cognate receptors for EG-VEGF and Bv8 are 80–90% identical GPCRs of the neuropeptide Y receptor class (19, 20). ACE cells express both R-1 and R-2 transcripts (20), and, putatively, both proteins. We conducted ISH studies to localize receptor expression in the testis. Initial studies with distinct ISH probes generated to the 3′ untranslated regions of the transcripts yielded no signal. Thereafter, we used an extended ISH probe that included coding sequence of mouse R-1. This resulted in a specific signal that was restricted to the endothelial cells of the interstitium (Fig. 5). The sense probe was devoid of detectable signal (data not shown). Because the probe could hybridize with both receptors, we established the identity of the transcript through the use of TaqMan analysis with distinct primer and probe sets. In mouse, both R-1 and R-2 are present at equivalent levels. Data from the human testis indicate that R-1 is more abundant. Overall, these receptors are expressed most predominantly in testis and brain (19, 20).

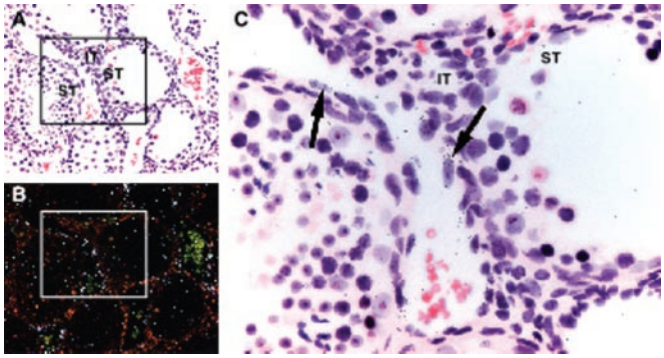

Fig. 5. Bv8 receptor expression is restricted to endothelial cells of the testis interstitium. (*A* and *B*) ISH with a probe derived from an mR-1 generated specific signal in the interstitium of the mouse testis. (*C*) Signal is localized to capillary endothelial cells, which are indicated by arrows. *A* and *B* are light- and dark-field images, respectively, and *C* is a higher magnification of the boxed area.

Discussion

The present studies demonstrate that Bv8, related to the recently characterized EG-VEGF, is highly conserved between human and mouse with respect to gene structure, promoter, and primary amino acid sequence. Additionally, at the level of bioactivity, Bv8 is comparable to EG-VEGF in a variety of assays. Recombinant Bv8 promotes proliferation, survival, and chemotaxis of ACE cells to the same extent as does EG-VEGF. Furthermore, Bv8, like EG-VEGF (or VEGF), induced a potent angiogenic response in the testis. A striking difference between these two molecules lies in the very distinct expression patterns in the human testis. EG-VEGF expression is restricted to the testosterone-producing Leydig cells (15). In contrast, Bv8 mRNA is highly expressed in and restricted to the primary spermatocytes in mice and humans. The related expression patterns of Bv8 are consistent with the conservation in promoter sequences. The high expression of Bv8 in the seminiferous tubule may relate to the hypoxic conditions existing within this structure.

Although the physiological role of Bv8 within the seminiferous tubules remains to be determined, it is known that the testis exhibits the highest endothelial cell turnover among the noncyclic tissues. Additionally, the testis, together with the epididymis, is a site of inflammation (32, 33) in response to primary or secondary bacterial and viral infections. In this context, we propose that Bv8 (and hEG-VEGF), in conjunction with VEGF and other cytokines, functions not only as an angiogenic mitogen to promote growth and survival of the testicular interstitial vessels, but also as a mediator of the inflammatory response. Bv8 may also perform this function in the context of the epididymis.

Most endocrine glands contain endothelial cells with specialized membrane microdomains referred to as fenestrae (34). EG-VEGF (10) or VEGF (35) promoted the formation of fenestrae in ACE

cultures. In the mouse, although Bv8 and VEGF are expressed in the testis, the endothelial cells do not exhibit this phenotype (36, 37), indicating that *in vivo* there is a greater level of complexity to regulate or determine this ultrastructural feature. In the human testis, endothelial cells within the lamina propria of the seminiferous tubule exhibit fenestrae (38). Therefore, it is tempting to speculate that EG-VEGF and VEGF expressed in the Leydig cells, along with Bv8 expressed in the tubule potentially contribute to the development of this phenotype.

Whereas the expression of Bv8 in an avascular compartment may appear contradictory with an angiogenic activity, Bv8 receptors are expressed in vascular endothelial cells that reside in the interstitial space. Also, delivery of exogenous Bv8 or EG-VEGF to the testis resulted in a potent angiogenic response. We do not exclude the idea that Bv8 potentially has other functions in the context of the male reproductive tract. Although primary spermatocytes express Bv8 mRNA, the molecule may potentially remain associated with the maturing sperm. $Bv8_{102}$ may be proteolytically processed and released as sperm mature, as previously suggested (26). The availability of reagents, including antibodies and the *Bv8* gene-targeted mouse, should permit further studies of the molecular function in the testis and during sperm maturation and fertilization.

Bv8 and EG-VEGF appear to share two GPCRs, referred to as Bv8/EG-VEGF/prokineticin R-1/I5E and R-2/ZAQ (19, 20). Here, we report the localization of these transcripts to the endothelial cells of the testicular interstitium. It is interesting that two highly related receptors exist within the testis and within a primary endothelial cell type (20). Gene-targeting studies may reveal unique roles or functional compensation in the distinct tissue contexts.

Although our principal focus has been the role of Bv8 in the testis, the major site of expression, we and others (16–20), have noted expression of both Bv8 and its two receptors in the brain. We did not detect Bv8 expression in the human brain by either Northern blot analysis or ISH studies. However, TaqMan analyses revealed a low signal in human and mouse brain. Cheng *et al.* (18) reported restricted expression of mBv8 within the suprachiasmatic nucleus, and other discrete brain regions, also indicative of a relatively low abundance message. Although EG-VEGF/Bv8 receptor expression within neurons has been evaluated (17, 18), expression by brain endothelial cells remains undefined. Intriguingly, in our initial report of EG-VEGF bioactivities, we noted a weak mitogenic effect in primary brain capillary endothelial cells (10). In addition to having direct effects on neurons, Bv8 may also function as an angiogenic factor in the brain. Potentially, Bv8 induction by hypoxic conditions may serve to protect both neurons and endothelium. Notably, VEGF appears to mediate neurotrophic effects through binding neuropilin-1 (39). Together, these findings emphasize the emerging molecular analogies between angiogenesis and neuronal sprouting and survival.

In conclusion, we have identified Bv8 as an angiogenic factor related to EG-VEGF. We propose that Bv8, in conjunction with EG-VEGF, VEGF, and other cytokines expressed in the testis, functions to maintain the vessels of the testis, and in part, protect the male germ cells. Overexpression of VEGF in the mouse testis resulted in an arrest of spermatogenesis (40). It will be interesting to assess whether overproduction of molecules such as Bv8 may also contribute to the pathophysiology of male infertility.

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