Overexpression of VEGF in Testis and Epididymis Causes Infertility in Transgenic Mice: Evidence for Nonendothelial Targets for VEGF

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Abstract. Vascular endothelial growth factor (VEGF) is a key regulator of endothelial growth and permeability. However, VEGF may also target nonendothelial cells, as VEGF receptors and responsiveness have been detected for example in monocytes, and high concentrations of VEGF have been reported in human semen. In this work we present evidence that overexpression of VEGF in the testis and epididymis of transgenic mice under the mouse mammary tumor virus (MMTV) LTR promoter causes infertility. The testes of the transgenic mice exhibited spermatogenic arrest and increased capillary density. The ductus epididymidis was dilated, containing areas of epithelial hyperplasia. The number of subepithelial capillaries in the epididymis was also increased and these vessels were highly permeable as judged by the detection of extravasated fibrinogen products. Intriguingly, the expression of VEGF receptor-1 (VEGFR-1) was detected in certain spermatogenic cells in addition to vascular endothelium, and both VEGFR-1 and VEGFR-2 were also found in the Leydig cells of the testis. The infertility of the MMTV-VEGF male mice could thus result from VEGF acting on both endothelial and nonendothelial cells of the male genital tract. Taken together, these findings suggest that the VEGF transgene has nonendothelial target cells in the testis and that VEGF may regulate male fertility.

Key words: VEGF • VEGF receptors • spermatogenesis • fertility • transgenic mice

Vascular endothelial growth factor (VEGF)[1] is a major regulator of blood vessel growth and permeability (for review see ref. 13). Its essential role in the development of the vasculature is emphasized by the finding that the loss of even a single VEGF allele results in embryonic lethality (5, 11). VEGF is also important during postnatal life in physiological angiogenic processes such as corpus luteum vascularization (12), and it has been strongly implicated in tumor angiogenesis. VEGF stimulates several stages of blood vessel formation including endothelial degradation of the underlying basement membrane, migration into the surrounding tissue, proliferation, and tube formation. It also acts as a survival factor for newly formed vessels (2). VEGF exerts its activities through binding to two receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). These receptors are expressed almost exclusively on endothelial cells, with a few exceptions such as VEGFR-1 in monocytes where it mediates chemotaxis (1, 7). Despite their structural similarity, the two VEGF receptors are functionally different: VEGFR-2 is required for the development of the endothelial lineage whereas VEGFR-1 plays a role in the organization of the vascular endothelium (14, 29). In vitro experiments have indicated that VEGF-induced proliferation is mainly mediated by VEGFR-2, whereas VEGFR-1 may be capable of mediating a migratory response (1, 7, 28, 31).

Recent findings have alluded to a novel role for VEGF in male fertility. VEGF has been shown to be expressed by several cell types in the male genital tract including Leydig and Sertoli cells of the testis, certain epithelial cells and peritubular cells of the epididymis, and the epithelium of the prostate and seminal vesicle (4, 9, 10). Concentration of VEGF in semen is very high, ~300 pM, which is well above the levels measured in tumor effusions or serum (4).

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1. *Abbreviations used in this paper:* MMTV, mouse mammary tumor virus; RT, room temperature; TG, transgenic; VEGF, vascular endothelial growth factor; VEGFR-1, VEGF receptor-1; vWF, von Willebrand factor; WT, wild-type.

The target cells and exact role of VEGF in the male genital tract are currently unknown. Both types of VEGFRs are expressed in testicular microvasculature and VEGFR-2 has been detected also in vascular endothelium of the epididymis (9, 10). The function of VEGF in the testis and epididymis may be associated with vascular permeability, as neither of these tissues are sites of active angiogenesis in the adult. Indeed, topical application of VEGF into the epididymis has been recently shown to induce fenestrations and transendothelial gaps in capillaries (10).

To investigate the effects of VEGF in vivo, we have generated transgenic mice expressing VEGF under the mouse mammary tumor virus (MMTV) LTR promoter. The MMTV promoter has been widely used to target transgene expression into the mammary gland, but it has been reported to be active also in certain other tissues including testis, epididymis, seminal vesicle, and prostate (3, 15). Transcriptional activity of MMTV is induced by steroid hormones including glucocorticoids, progestins, androgens, and mineralocorticoids, and prolactin also contributes to high expression (for review see ref. 15). In this study we have investigated the effect of MMTV LTR-driven VEGF overexpression in the testis and epididymis. Analysis of the mammary gland phenotype of MMTV-VEGF female mice will be presented elsewhere.

Materials and Methods

Generation of the MMTV-hVEGF165 Transgenic Mice

The MMTV-hVEGF165 transgene vector was constructed by cloning the MMTV-LTR sequence from pA9 (19) as a BamHI-BglII fragment into pGEM3Zf(+) (Promega, Madison, WI). hVEGF-165 cDNA (bp 57–638 [22]) was then added as a BamHI fragment. Finally, the bovine growth hormone polyadenylation signal was PCR amplified from pcDNA3 (Invitrogen, Carlsbad, CA) and cloned as an EcoRI fragment into the vector. A HindIII-NdeI fragment of the resulting construct was microinjected into fertilized oocytes of the FVB/N strain of mice, and the injected zygotes were transplanted into oviducts of pseudopregnant C57BL/6xDBA/2J hybrid mice. The resulting founder mice were analyzed for the presence of the transgene by PCR of tail DNA using primers 5′-CTGCA-GATATCCATCACACTGG-3′ and 5′-ACCTACTCAGACAATGC-GATGC-3′. The tail DNAs were also subjected to endonuclease digestion, Southern blotting, and hybridization analysis using the hVEGF-165 cDNA fragment as the probe.

RNA Isolation and Northern Blot Analysis

For total RNA preparation, tissues were frozen in liquid nitrogen, pulverized with Mikro-Dismembrator U (B. Braun Biotech International, Melsungen, Germany) and the RNA was isolated using the guanidium thiocyanate-method (6). 10 μg total RNA samples were size fractionated on 1.0% formaldehyde-agarose gels, transferred to Nytran® nylon membrane (Schleicher & Schuell, Dassel, Germany), UV cross-linked, and then hybridized with the hVEGF-165 cDNA probe. The rGAPDH cDNA probe was used as an internal control for equal loading. The probes were labeled with [α³²P]dCTP (3,000 Ci/mmol; Amersham Int. plc, Buckinghamshire, UK) by random priming. Prehybridizations and hybridizations were performed at 42°C in a solution containing 50% formamide, 5× Denhardt solution, 5× SSPE, 0.5% SDS, and 200 μg/ml salmon sperm DNA. The filters were washed once for 30 min at room temperature and twice for 10 min at 65°C with 1× SSC, 0.1% SDS, and then exposed to Fuji Medical X-ray film after quantitation by phosphorimager analysis (Bio-Imaging Analyzer BAS1500; Fuji, Tokyo, Japan).

In Situ Hybridization

For in situ analysis the tissues were fixed in paraformaldehyde overnight (o/n) at 4°C and dehydrated in an increasing ethanol series followed by xy-

lene and then embedded in paraffin. 6-μm sections were placed on a layer of diethyl pyrocarbonate-treated water on the surface of glass slides pretreated with 2% 2-aminopropyltriethoxysilane. The antisense and sense hVEGF cRNA probes were synthesized from linearized pGEM-3Zf(+) plasmids containing the hVEGF cDNA insert in both orientations, using T7 polymerase and [³⁵S]dUTP (Amersham). The template for mVEGFR-1 probe containing the bp 1,438–2,090 of the mVEGFR-1 (GenBank/EMBL/DDBJ accession number L07297) was generated by PCR and cloned into pCR2.1-TOPO vector (Invitrogen) in both orientations. Antisense and sense probes were generated using T7 polymerase. The mVEGFR-2 probes were prepared as previously described (20). In situ hybridization was performed according to Wilkinson et al. (33, 34) with the following modifications: the sections were treated with 10 μg/ml of proteinase K for 15 min at room temperature (RT), the alkaline hydrolysis of the probes was omitted, and the high stringency wash was for 60 min in 65°C in a solution containing 30 mM DTT, 2× SSC, and 50% deionized formamide. The sections were covered with NTB-2 emulsion (Eastman-Kodak, Rochester, NY), exposed at 4°C for 35 d, developed, and then stained with hematoxylin.

Immunohistochemistry

For immunohistochemical analysis the tissues were fixed in paraformaldehyde for 15 h at 4°C and embedded in paraffin after dehydration. 4-μm sections were deparaffinized, dehydrated, and then incubated with 0.25 mg/ml trypsin (Difco, Detroit, MI) in 0.05 M Tris buffer, pH 7.8, containing 9 mM CaCl₂ for 20 min at 37°C for antigen retrieval before the blocking of the endogenous peroxidase activity with 3% H₂O₂ in methanol for 10 min at RT. The immunohistochemical staining for von Willebrand Factor (vWF) was done using the Tyramide Signal Amplification system (TSA®; New England Nuclear Life Science Products, Boston, MA) according to the manufacturer's instructions. The sections were incubated with 7.5 μg/ml polyclonal rabbit anti-hvWF antibody (Ab) (A0082; Dako, Carpinteria, CA) at 4°C o/n. For negative controls the section was incubated in the blocking reagent without the primary Ab. The incubation with the secondary Ab was for 30 min at RT with 7.5 μg/ml of biotinylated goat anti–rabbit IgG (Vector Laboratories, Burlingame, CA). After the amplification steps the peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma Chemical Co., St. Louis, MO) in 0.05 M sodium acetate buffer, pH 5.0, and the sections were counterstained with hematoxylin. The testicular capillaries stained for vWF were counted from 10 high-power microscopic fields. The average numbers and standard deviations were then derived from the analysis of three transgenic and control mice. Immunohistochemical detection of fibrinogen and its degradation products was performed similarly except that 10% goat serum was used for blocking and the polyclonal rabbit anti-human fibrinogen Ab (A0080; Dako) was used as the primary Ab at 9 μg/ml. Instead of the TSA amplification method, the sections were incubated for 30 min at RT with the Vectastain Elite ABC reagent (Vector Laboratories) before the color development.

Analysis of the Seminiferous Tubules by Transillumination and Squash Preparations

Seminiferous tubules were dissected and photographed under a transillumination dissection microscope, and the spermatogenic cells were identified from live cell squash preparations (25).

Results

MMTV-VEGF Transgenic Male Mice Are Infertile

To study the effect of VEGF overexpression in transgenic mice, the cDNA coding for the 165-amino acid isoform of hVEGF was expressed under the MMTV promoter. Nine different MMTV-VEGF transgenic lines were obtained, with copy numbers ranging from <1 (mosaic) to 18 as judged by Southern blotting (data not shown). The MMTV-VEGF mice appeared healthy and did not exhibit any macroscopic physical aberrations or reduction in body weight. To confirm transgene expression, RNA samples from different tissues were analyzed by Northern blotting
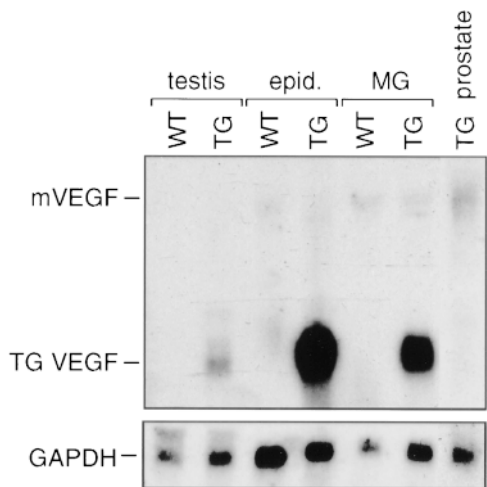
Figure 1. Expression of the VEGF transgene mRNA in the epididymis and testis. Total RNA was prepared from the testis, epididymis, and prostate of MMTV-VEGF transgenic mice and WT control mice and then 10-μg samples were analyzed by Northern blotting using the hVEGF-165 cDNA fragment as a probe (*top*). Mammary gland (*MG*) RNA of MMTV-VEGF female mice was used as a positive control. Signals corresponding to the VEGF transgene mRNA (*TG VEGF*) and the endogenous mouse VEGF mRNA are shown. The filter was stripped and reprobed for GAPDH to check RNA loading (*bottom*).

(Fig. 1). As expected, high levels of the VEGF transgene mRNA were detected in mammary glands of lactating females. Interestingly, the transgene was expressed at comparable levels in the epididymis and to a lesser extent in the testis, whereas no expression was detected in the prostate (Fig. 1) or seminal vesicle (data not shown).

As studies on human testis and epididymis have recently suggested that VEGF may have a role in the male reproductive tract (4, 9, 10), we investigated the effect of VEGF overexpression on the fertility of the MMTV-VEGF mice. MMTV-VEGF males of five transgenic lines which differed in their level of transgene expression in the epididymis as judged by Northern blotting (data not shown) were kept in continuous breeding from 8 wk to 6 mo of age. Strikingly, the MMTV-VEGF males sired no offspring during this period, whereas other transgenic and nontransgenic mice as well as MMTV-VEGF females in the same facility reproduced normally.

The Ductus Epididymidis of MMTV-VEGF Mice Is Dilated and Hyperplastic

Comparison of the 6-mo-old MMTV-VEGF males with their wild-type (WT) littermates indicated that their epididymides were enlarged and swollen, whereas no apparent differences in testes, prostate, seminal vesicle, or other internal organs were observed. Histological examination (Fig. 2, *A–E*) confirmed that the ductus epididymidis was dilated, especially in caput and corpus epididymidis. The epithelium of the caput epididymidis was reduced in height, reminiscent of that found normally in the cauda region. The connective tissue septa between the segments contained enlarged blood vessels. All transgenic animals

exhibited epithelial hyperproliferation dividing the duct into multiple narrow sublumens in proximal cauda epididymidis, and in some animals hyperplasia was observed also in the caput region. These hyperplastic areas were interspersed and surrounded by an increased number of subepithelial capillaries as evidenced by the presence of red cells and the vWF staining of the endothelial cells (Fig. 2, *E* and *F)*. Staining using antibodies to fibrinogen, which has been used to detect increased capillary permeability resulting in fibrin deposition in tissues (24), indicated leakage of this plasma protein in between the epithelial cells and on the luminal side of the ductal epithelium (Fig. 2, *G* and *H)*. Spermatozoa could be seen only in some ductal sections preceding the hyperplastic zones.

VEGF Overexpression in the Ductal Epithelium Upregulates the Expression of VEGFRs

Expression the VEGF transgene in the epididymis was further localized by in situ hybridization. As shown in Fig. 2 *I*, VEGF mRNA was strongly expressed in the epithelium of the ductus epididymidis, the proximal caput epididymidis and the hyperplastic areas of cauda epididymidis showing the highest level of transgene expression. To investigate the possible target cells for VEGF, in situ hybridization was performed using VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) riboprobes. In WT control mice, VEGFR-1 was expressed in the endothelium of the interstitial blood vessels throughout the epididymis, whereas no expression of VEGFR-2 could be detected (Fig. 2, *N* and *L)*. In contrast, both receptors were strongly upregulated in the epididymides of TG animals (Fig. 2, *K* and *M)*. Their expression followed closely that of VEGF, but the patterns were not overlapping: whereas VEGF was confined to the inner epithelium of the duct, the receptors were expressed in the outer rim. This difference was clearly visible in the hyperplastic areas where the receptor signals could be seen also in presumptive endothelial cells inside the hyperplastic nodules, surrounded by the epithelial VEGF signal. VEGFR-1 expression in the walls of the interstitial blood vessels was similar to that in the WT animals.

Aberrant Spermatogenesis in MMTV-VEGF Mice

Because of the lack of spermatozoa in the ductus epididymidis, the testes of MMTV-VEGF males were analyzed in detail. The seminiferous tubules were first examined in freshly isolated living condition by transillumination. Since tubular segments containing maturation phase spermatids appear dark due to the DNA compaction, this technique can be used to obtain a rapid overview of the spermatogenic development (25). Although the MMTV-VEGF mice had some normal-looking tubules, the majority of the tubules appeared homogeneously pale, lacking the normal spermatogenic wave (Fig. 3, *top*). Squash preparations of these segments demonstrated that spermatogenesis was interrupted at the elongation phase of the spermatids (Fig. 3, *bottom*). Neither elongated spermatids or spermatozoa were observed, but somatic Sertoli cells, spermatogonia, meiotic cells, and round spermatids appeared normal. We also observed many phase-negative cellular spheres, which have been shown to correspond to apoptotic bodies (16). Some tubules contained heavily light absorbing material
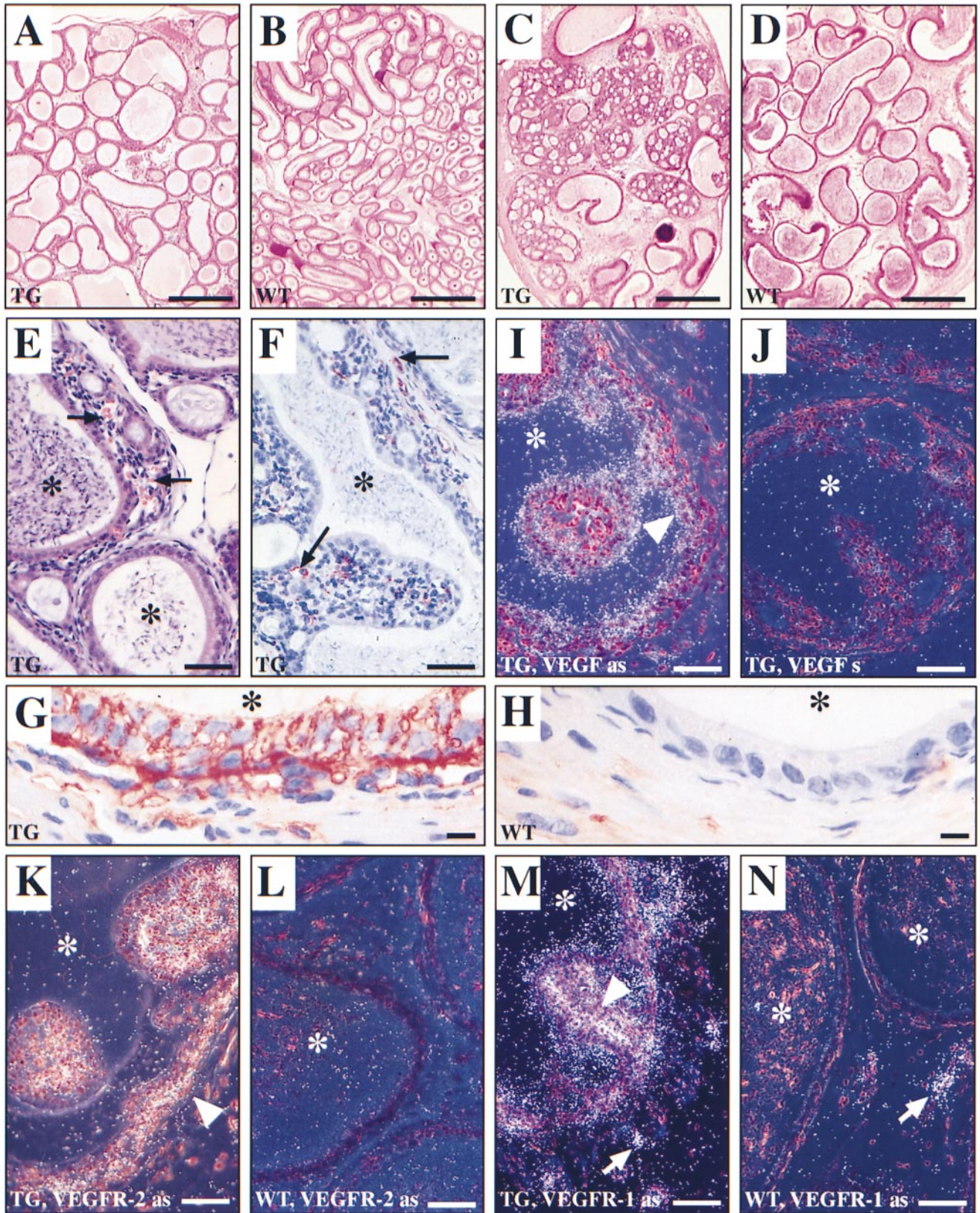
Figure 2. Histological comparison of epididymides of 6-mo-old MMTV-VEGF transgenic and WT control mice (*A–H*), and localization of expression of the VEGF transgene and VEGFRs by in situ hybridization (*I–N*). The ductus epididymidis of TG mice is dilated and devoid of spermatozoa in the caput region (*A*) and exhibits epithelial hyperplasia in the cauda epididymidis (*C*). Caput and cauda epididymidis of a WT control mouse are shown in *B* and *D*, respectively. Higher magnification of the hyperplastic epithelium in the cauda epididymidis of the MMTV-VEGF mice (*E*) reveals the presence of interspersed capillaries (*arrows*). Immunohistochemical staining of these capillaries for vWF is shown in *F*. The fibrinogen staining in the same area (*G*) indicates that the capillary permeability is in-
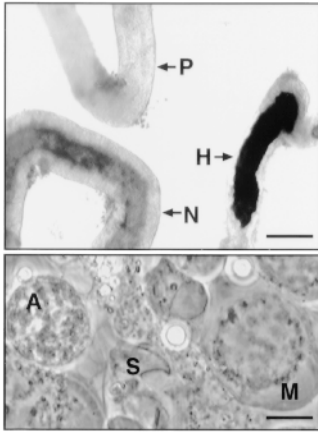
Figure 3. Aberrant spermatogenesis in MMTV-VEGF transgenic mice. Transillumination analysis of seminiferous tubules dissected from the testis of a 6-mo-old MMTV-VEGF mouse (*top*) indicated that whereas some tubules appeared normal (*N*), the majority were homogeneously pale (*P*), lacking the normal spermatogenic wave, and some contained heavily light absorbing hard material (*H*). Squash preparation of the pale segment (*bottom*) shows that the spermatogenesis is interrupted at the elongation phase of the spermatids. Normal meiotic cell (*M*), step 9 spermatid (*S*), and apoptotic spermatocyte (*A*) are indicated. Bars: (*top*) 0.25 mm; (*bottom*) 10 μm.

that appeared to have a very hard consistency, which did not permit a normal analysis of living cells in the squash preparations (Fig. 3, *top*).

Histological analysis of the testes of the 6-mo-old MMTV-VEGF mice confirmed that whereas some seminiferous tubules appeared relatively normal, others contained vacuoles and disorganized seminiferous epithelium with few spermatogenic cells (Fig. 4, *A–D*). The absence of developing spermatids and spermatozoa was clear and numerous degenerating cells were observed in the atrophic tubules. In contrast, the somatic Sertoli and Leydig cells appeared normal. The rete testis was remarkably dilated in the transgenic animals when compared with that of the WT controls. The capillary density was increased by 60 ± 20% as judged by counting of blood vessels in sections stained for the vWF. Also, many of the capillaries of the TG testes were enlarged when compared with the controls.

Expression of the VEGF Transgene in Spermatogenic Cells and VEGFRs in Nonendothelial Cells

The sites of VEGF transgene expression in the testis were localized by in situ hybridization. The VEGF signal was found inside the seminiferous tubules and it seemed to be restricted to step 1–12 spermatids, whereas no expression was observed in the Sertoli cells (Fig. 4, *E–G*). VEGFR expression was subsequently studied by in situ hybridization in order to identify potential VEGF-responsive cells. Testes of WT control mice showed no hybridization with the VEGFR-2 riboprobe but, interestingly, strong VEGFR-1 signals were observed in stage VIII seminiferous tubules

(Fig. 4, *K–M*). In these tubules the grains accumulated specifically over midpachytene spermatocytes and round (step 8) spermatids. In MMTV-VEGF mice, low levels of VEGFR-1 signal were seen also in round spermatids of the other stages. Interestingly, the expression of both VEGFR-1 and -2 was strongly upregulated in the interstitial tissue of the testes of TG animals (Fig. 4, *H–J* and *N*). Careful analysis of the VEGFR signals in brightfield microscopy showed that the majority of the in situ silver grains were localized in nonendothelial cells identified as Leydig cells by their morphology and location (Fig. 4 *J*).

Discussion

In this work we present evidence that overexpression of VEGF in the testis and epididymis of transgenic mice under the MMTV promoter leads to upregulation of VEGF receptors in the endothelial cells, in certain spermatogenic cells, and in the Leydig cells, and causes infertility. The testes of MMTV-VEGF mice exhibited spermatogenic arrest and the ductus epididymidis was dilated, containing areas of epithelial hyperplasia. Although some of these effects are likely to be due to the action of VEGF on the vascular endothelium, our data suggests that VEGF may act also directly on nonendothelial cells including the Leydig cells and certain spermatogenic cells.

The MMTV-driven VEGF transgene was found to be expressed in round spermatids in seminiferous tubules and in the ductal epithelium of the epididymis. Although the latter finding conforms with the inducibility of the MMTV LTR promoter by androgens, the expression in step 1–12 spermatids (and not, for example, in Sertoli cells) is somewhat surprising as the androgen receptor is expressed in Sertoli cells at stages IV–VIII of the cycle, and in elongating step 11 spermatids in rat seminiferous epithelium (32). In mice, spermatogonia have also been reported to contain androgen receptors (36). The glucocorticoid receptor which could also potentially activate the MMTV LTR has only been observed in primary spermatocytes (27). To our knowledge the MMTV LTR transcriptional activity has not been previously localized by in situ hybridization in the testis (or epididymis), and the results presented here suggest that the regulation of the MMTV LTR is more complex than previously appreciated.

Interestingly, the expression of VEGFRs was not restricted to the vascular endothelium of the testis and epididymis (Table I). VEGFR-1 was expressed in stage VIII seminiferous tubules where it localized to midpachytene spermatocytes and round spermatids as judged by in situ hybridization. In MMTV-VEGF mice, low levels of VEGFR-1 signal were seen also in the round spermatids of other stages. Furthermore, both VEGFR-1 and -2 were observed in the Leydig cells in the MMTV-VEGF mice,

creased in the TG mice when compared with the WT mice (*H*). *Asterisks*, lumen of the ductus epididymidis. The VEGF transgene is expressed in the ductal epithelium (*arrowhead*) as judged by in situ hybridization (*I*, antisense and *J*, sense hVEGF probe). In contrast, the VEGFR-2 antisense probe localizes to the outer rim of the duct (*arrowhead*) and inside the hyperplastic nodules in cauda epididymidis of MMTV-VEGF mice (*K*), whereas no hybridization signal is seen in the WT control mice (*L*). VEGFR-1 is expressed around the ductus epididymidis and inside the hyperplastic nodules (*arrowhead*) in TG mice (*M*) and it is also found in interstitial blood vessels (*arrow*). In WT mice (*N*) the mVEGFR-1 antisense probe recognizes only the interstitial blood vessels. Bars: (*A–D*) 500 μm; (*E, F, I, J,* and *K–N*), 50 μm; (*G* and *H*), 10 μm.
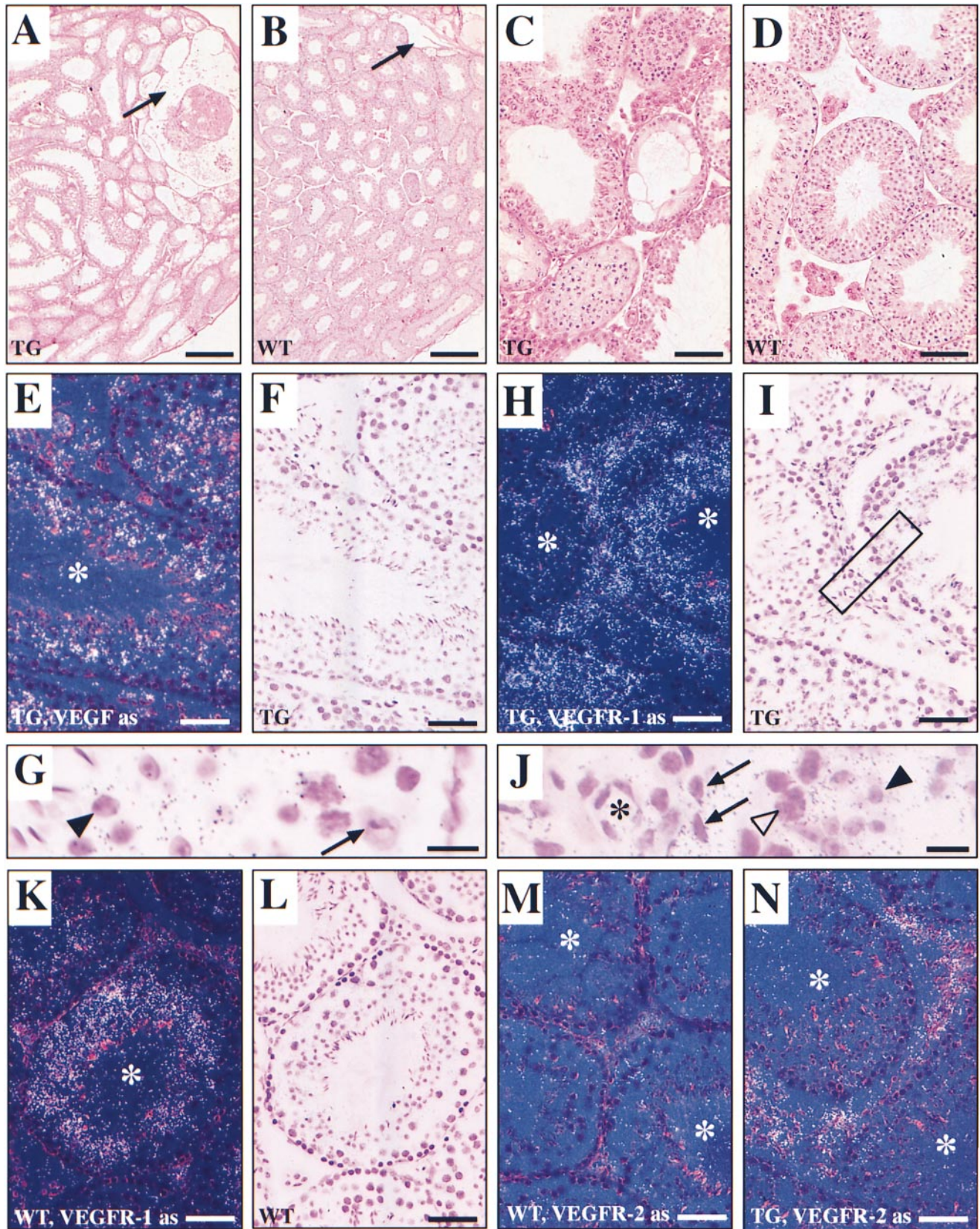
Figure 4. Histological analysis of testes (*A–D*) and localization of expression of the VEGF transgene and VEGFRs by in situ hybridization (*E–L*). Comparison of testes from a 6-mo-old MMTV-VEGF transgenic (*A* and *C*) and WT control mouse (*B* and *D*) indicates that in the transgenic animals the rete testis (*arrows*) is dilated and the seminiferous tubules contain vacuoles and disorganized seminiferous epithelium. The VEGF transgene is expressed in round spermatids (*arrowhead*) but not in Sertoli cells (*arrow*) as judged by in situ hybridization with the hVEGF antisense probe (*E*, dark field; *F* and *G*, bright field). *Asterisk*, lumen of the seminiferous tubule. VEGFR-1

Table I. Summary of the Cell Types Expressing VEGF and Its Receptors in the Testis and Epididymis of WT and MMTV-VEGF Transgenic Mice as Judged by In Situ Hybridization

	Testis	Epididymis
VEGF	Round spermatids (TG only)	Ductal epithelium (TG only)
VEGFR-1	Round spermatids	Endothelial cells of interstitial blood vessels
	Midpachytene spermatocytes	
	Leydig cells (TG only)	Endothelial cells of capillaries within the hyperplastic epithelium (TG only)
VEGFR-2	Leydig cells (TG only)	Endothelial cells of capillaries within the hyperplastic epithelium (TG only)

suggesting that they were upregulated by the overexpression of VEGF. This upregulation could depend on the direct interaction of VEGF with small numbers of preexisting VEGFRs in these cells, similar to what has been described for autocrine VEGFR-2 upregulation in brain endothelial cells exposed to hypoxia (21). In keeping with our findings, VEGFR-1 and -2 have been recently detected in the Leydig cells of human testes by immunohistochemistry (9).

The epididymides of all MMTV-VEGF mice exhibited epithelial hyperplasia in the cauda epididymidis. This regional specificity may reflect the fact that the segments of epididymis differ from each other not only morphologically but also functionally and biochemically (18, 26). It is thus possible that the epithelium of the caudal part is more responsive to the effects of the transgene. The level of transgene expression was also higher in the hyperplastic area than in the neighboring parts, suggesting that this area may be more sensitive to signals activating the MMTV promoter. These VEGF overexpressing areas exhibited abnormal growth and permeability of capillaries, which may be responsible for the hyperplasia of the ductal epithelial cells. For example, the VEGF-stimulated endothelium could provide mitogens for the epithelial cells in a paracrine manner. On the other hand, the extravasation of plasma proteins could contribute to the enhanced proliferation of the epithelial cells. The capillaries associated with the epithelium expressed high levels of VEGFR-2 (and VEGFR-1), whereas the nearby interstitial capillaries remained VEGFR-2–negative and seemingly unaffected. VEGF overexpression therefore seems to have rather short-range effects on the vascular network in the epididymis, targeting mainly subepithelial capillaries. Also in the testis VEGF mainly increased the capillary density, in contrast to other VEGF overexpression models where large vessels or vascular sacs were seen (2, 35). It is possible that the vascular effects of VEGF depend on the tissue context where it is expressed.

The infertility of MMTV-VEGF male mice is probably due to effects of VEGF on both the testis and the epididymis. The finding of VEGFR-1 receptors in midpachytene spermatocytes and round spermatids suggests that VEGF can act directly on spermatogenic cells. The expression in the round spermatids which are only beginning to elongate is of particular interest as the observed spermatogenic arrest occurred at this point. VEGF could affect spermatogenesis also by acting on the Leydig cells, which are the main source of testosterone. However, the testosterone levels in testis homogenates and serum of MMTV-VEGF mice were normal (data not shown). The vascular effects of VEGF overexpression in testis could also contribute to the infertile phenotype, as the increased capillary density and size are likely to elevate the testicular temperature.

VEGF overexpression in the epididymis was associated with increased vascular permeability as evidenced by extravasation of fibrinogen resulting in intraepithelial and intraluminal fibrin deposition. The increased permeability may have important consequences especially in the caput region, where a significant portion of the luminal fluid from the testis is normally reabsorbed by the ductal epithelium which is surrounded by a dense capillary network (30). The reabsorption of fluid serves to concentrate the sperm, improving its survival and maturation in the epididymis. The importance of this function was recently emphasized by the finding that oestrogen receptor-α knockout (ERKO) mice are infertile due to defective epididymal reabsorption (17). Notably, the ERKO mice exhibited dilated rete testis and long-term atrophy of testes due to back-pressure of the luminal fluid (8). The aberrations seen in the testes of MMTV-VEGF males could thus be, at least partly, due to the effect of VEGF on vascular permeability in the epididymis. In keeping with this possibility, VEGF has been recently shown to increase the number of fenestrations, transendothelial gaps, and transcytotic vesicles in the endothelium of epididymal capillaries (10). Defective fluid reabsorption could lead to the observed swelling of the ductus epididymidis. Alternatively, the swelling could be a consequence of ductal occlusion by the hyperproliferation of the caudal epithelium.

Taken together, our present results show that overexpression of VEGF under the MMTV LTR promoter in the testis and epididymis of transgenic mice results in infertility, probably via the effects of VEGF on both endothelial and nonendothelial cells of the male genital tract. It is striking that although over 30 different transgenes including various (proto)oncogenes have been expressed under

antisense probe recognizes midpachytene spermatocytes and round spermatids in stage VIII seminiferous tubules of the WT mice (*K*, dark field; *L*, bright field) and also Leydig cells in the testis of the MMTV-VEGF mice (*H*, dark field; *I*, bright field). The area enclosed within the rectangle in *I* is shown at higher magnification in *J*. Leydig cell (*arrow*), round spermatid (*arrowhead*), midpachytene spermatocyte (*open arrowhead*), and a capillary (*asterisk*) are indicated. VEGFR-2 signal is detected in the Leydig cells of the MMTV-VEGF mice (*N*), whereas the testis of a WT control mouse does not show any hybridization with the VEGFR-2 antisense probe (*M*). Bars: (*A* and *B*), 500 μm; (*C* and *D*) 100 μm; (*E*, *F*, *H*, *I*, and *K*–*N*) 50 μm; (*G* and *J*) 10 μm.

the MMTV LTR, only one of them, the constitutively active oncogenic mutant of the Neu tyrosine kinase receptor, caused epithelial hyperplasia of the epididymis resulting in infertility (3, 23). However, the MMTV-neu males were fertile until 3–4 mo of age (3), whereas the MMTV-VEGF males never sired any litters. The dramatic phenotype of the MMTV-VEGF males is of interest as recent studies have reported detection of VEGF and its receptors in the human testis, epididymis, seminal vesicle, and prostate tissues, and high VEGF concentrations in semen (4, 9, 10). Although the exact functions of VEGF in the male genital tract remain to be elucidated, it is likely to play an important role in male fertility. It should be noted that systemic inhibition of VEGF function in female rats using a recombinant soluble VEGFR-1 curtails corpus luteum development (12). The striking finding that VEGF has nonendothelial target cells in the testis should make it possible to study and perhaps even to manipulate the reproductive functions of VEGF in males.

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