Multiple Developmental Roles of VEGF Suggested by a LacZ-Tagged Allele

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Vascular endothelial growth factor (VEGF) is an angiogenic factor and a potent stimulator of microvascular permeability. It is a mitogen specific for endothelial cells. The expression of VEGF and its two receptors, Flk-1 and Flt-1, is pivotal for the proper formation of blood vessels in embryogenesis as shown by gene-targeting experiments. Interestingly, the loss of even a single allele of VEGF led to embryonic lethality between day E9.5 and day E10.5 in the mouse. To assess the role of VEGF during embryonic development we decided to tag VEGF expression with LacZ, by inserting an IRES (internal ribosome entry site)-LacZ reporter cassette into the 3' untranslated region of the gene. This alteration enabled us to monitor VEGF expression throughout embryonic development at single-cell resolution. β-Galactosidase expression from the altered VEGF locus was first observed prior to gastrulation and was detectable at all stages of vascular development in the embryo. Later, the specific cellular distribution and the level of VEGF expression indicated its pleiotropic role in development. High expression levels seemed to be associated with vasculogenesis and permeability, whereas lower levels were associated with angiogenesis and cell migration. In addition, we found VEGF expression in a subtype of endothelial cells present in the endocardium. We believe that the LacZ-tagged allele we have generated offers a precise means of detecting VEGF expression under a variety of physiological and pathological conditions.© 1999 Academic Press

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INTRODUCTION

In all tissues, a functional vasculature is indispensable for growth, differentiation, and maintenance. During embryonic development, blood vessels are initially formed by in situ differentiation of endothelial precursors (angioblasts), followed by their assembly into tubes, a mechanism known as vasculogenesis (Risau and Flamme, 1995). Later in the embryo and adult, a second mechanism called angiogenesis also operates when a complex vascular network arises by sprouting and remodeling of new capillaries from the pre-existing blood vessels (Risau, 1997). Angiogenesis also plays a role in blood vessel formation in pathological situations such as wound healing, retinopathy, and tumor growth (Folkman and D’Amore, 1996). Endothelial cells play a key role in both of these processes. The differentiation and proliferation of the endothelium is a complex process involving paracrine signals that act on endothelial cells via specific cell surface receptors (Augustin et al., 1994; Risau, 1995).

Vascular endothelial growth factor (VEGF) is essential for vascularization in both physiological and pathological situations (review in Ferrara and Davis-Smyth, 1997). It was initially purified from tumor cells as vascular permeability factor based on its ability to induce hyperpermeability of the tumor blood vessels (Senger et al., 1983). VEGF acts as both an endothelial cell-specific mitogen (Keck et al., 1989; Leung et al., 1989) and an angiogenic factor (Plouët et al., 1989) and possesses chemoattractant activities for endothelial and nonendothelial cells, including monocytes and osteoclasts (Clauss et al., 1990; Senger et al., 1996; Yoshida et al., 1996). VEGF is a dimeric glycoprotein that exists in three different isoforms of 120, 164, and 188 amino acids.
produced by alternative splicing of a single gene in the mouse (Shima et al., 1996). The most abundant isoforms are VEGF$_{120}$ and VEGF$_{164}$. The mitogenic potential of VEGF$_{164}$ is higher than that of VEGF$_{120}$ (Keyt et al., 1996; Wilting et al., 1996). All VEGF isoforms can bind either of two receptor tyrosine kinases, VEGFR1 (or Flt-1) or VEGFR2 (or KDR/Flik-1) (de Vries et al., 1992; Yamaguchi et al., 1993). Both of these receptors are present on endothelial cells but the biological activities that they transduce differ. VEGFR2 appears to be the primary receptor that mediates the mitogenic and chemotactic effects of VEGF on endothelial cells (Clauss et al., 1996; Waltenberger et al., 1994).

Embryos homozygous for the targeted disruption of VEGFR2/Flik-1 die around day E8.5 and fail to form blood islands and therefore lack mature endothelial and hematopoietic cells (Shalaby et al., 1995). The dual activity of Flik-1 on endothelial and hematopoietic cells was confirmed by chimeric analyses in mouse embryos which showed that VEGFR2/Flik-1 is required cell autonomously for endothelial cell differentiation and is also required, but not sufficient, for hematopoietic cell differentiation (Shalaby et al., 1997). VEGFR1/Flt-1 binds VEGF with high affinity; it has not been shown to transmit a mitogenic signal, but may be involved in cell migration (Barleon et al., 1996). The importance of VEGFR1 during embryonic development was demonstrated in gene targeting studies which show that embryos deficient for VEGFR1 die in utero at day E9.5 as a result of a disorganized vascular system associated with an overproduction of endothelial cells (Fong et al., 1995). VEGFR1 signal transduction does not seem to include the tyrosine kinase domain of the receptor since the disruption of this domain by gene targeting in the mouse does not affect normal embryonic development (Hiratsuka et al., 1998).

Recently, Soker et al. reported a third VEGF-receptor, neuropilin-1, which binds the VEGF$_{164}$ isoform and can potentiate VEGFR2 activity, acting as a coreceptor. Neuropilin-1 was first identified as a semaphorin/collapsin receptor involved in axon guidance (Soker et al., 1998). Overexpression of neuropilin results in cardiovascular defects in embryos, suggesting that the VEGF-neuropilin complex may play a role in normal embryonic vascular development (Kitsukawa et al., 1995).

The role of VEGF during development was investigated by gene targeting and reveals that the loss of a single allele is sufficient to impair vascular development. Embryos lacking a single functional VEGF allele die in utero at day E10.5 due to severe defects in the formation of the dorsal aorta and in yolk sac vessels (Carmeliet et al., 1996; Ferrara et al., 1996). Homozygous embryos present an even more severe phenotype, suggesting a tight dose-dependent regulation of embryonic vessel development by VEGF (Carmeliet et al., 1996). Moreover, the absence of VEGF in ES cells impaired tumor growth when those cells were injected under the skin of nude mice (Ferrara et al., 1996).

These data indicate that VEGF plays a central role in vascular development and suggest that VEGF regulation as well as its signaling pathways is complex due to the interplay of several isoforms and receptors. Thus, the systematic characterization of the cells that express VEGF will help to clarify the complexity of its function. Previous studies using RNA in situ hybridization have shown expression of VEGF during embryonic development at various stages and in numerous tissues (Breier et al., 1992; Dumont et al., 1995; Monacci et al., 1993; Peters et al., 1993). This expression was correlated with the expression of the two VEGF receptors, VEGFR1 and VEGFR2, in the adjacent endothelial cells (Dumont et al., 1995; Millauer et al., 1993), leading to the conclusion that VEGF may have pleiotropic activities, acting on the proliferation, differentiation, migration, and maturation of endothelial cells. As in situ hybridization can rarely provide single-cell resolution and antibodies suitable for immunohistochemistry are not available or would only detect VEGF bound to its receptors, we attempted to visualize VEGF expression by introducing a LacZ reporter gene into the 3' untranslated region of the endogenous VEGF locus by homologous recombination. The use of an internal ribosome entry site (IRES) in front of LacZ allows independent translation of both VEGF and the reporter from the same bicistronic mRNA produced by the targeted allele. This knock-in strategy provides an accurate readout of VEGF expression at the cellular level and at the same time circumvents the lethal haploinsufficiency of the gene.

Here, we report a very intricate regulation of VEGF throughout the entire process of embryonic development, including early implantation and late organogenesis. The complexity of expression points out not only the pivotal role of this growth factor in endothelial cell proliferation but also its possible other functions as chemoaattractant and/or survival factor for endothelial and other cell types. This approach also provides evidence against the generally accepted view that endothelial cells themselves do not express VEGF; a certain subtype of endothelial cells, i.e., the endocardium, and cells in the outflow tract of the heart were positive for VEGF. We suggest that this model can be used to investigate the induction of VEGF expression in physiopathological conditions in adult mice.

**MATERIAL AND METHODS**

**Construction of the Targeting Vector**

Murine genomic clones (strain 129) encompassing exon 4 to exon 8 of the VEGF gene (Shima et al., 1996) were used to construct the targeting vector pVEGF-LacZ-KI. The bacterial β-galactosidase gene (LacZ) was fused to a nuclear localization signal and an IRES sequence (Jang et al., 1988; Takeuchi et al., 1995). The neomycin selection marker, flanked by two identically oriented loxP sites (pLoxPneo) (Takeuchi et al., 1995), was introduced after the LacZ cassette. The IRES-NLS-LacZ-poly(A)-loxP-neo-loxP cassette was
ligated into the Smal site located in the 3' untranslated region (UTR) of VEGF 200 bp downstream of the stop codon.

**Generation of VEGF<sup>LacZ</sup>+/ES Cells and VEGF<sup>LacZ</sup> and VEGF<sup>LacZ<sub>Lneo</sub></sup> Mice**

The pVEGF-LacZ-KI vector was linearized using XhoI and electroporated into R1 ES cells as described previously (Nagy et al., 1993). Genomic DNA prepared from 154 G418-resistant ES cell clones was digested with BglII and screened by Southern blot using a 400-bp BglII-EcoRI probe encompassing the VEGF exon 3. Correct targeting was further confirmed by EcoRV digestion and using a 1-kb HindIII probe in the 3' UTR. Three independent clones (G3, D10, and F6) were aggregated with CD-1 morulae as described previously (Pirity et al., 1998). All analyses described further were performed on CD-1/129 hybrid background.

**Genotyping of Progeny**

To identify mice carrying the targeted allele PCR analysis was performed on ear punches or yolk sac samples. Oligonucleotides used here, VEGF-WT, 5'-ATGTGACAAGGCCAAGCGGTG-3', and VEGF-exon8, 5'-TGCCGATTTAGCGACGACA-3', giving a 300-bp band, identified the wild-type allele. To distinguish the two targeted alleles, different primers were used: Neo, 5'-CCACATACACTTCTTCTCAG-3', VEGF-exon8, amplifying a 285-bp band corresponding to the VEGF<sup>LacZ</sup>; and LacZ<sup>-</sup>, 5'-GCTAGGCTTCTTACAGAC-3', and VEGF-exon8, amplifying a 450-bp band for VEGF<sup>LacZ<sub>Lneo</sub></sup>.

**β-Galactosidase Staining of Embryos**

Embryos were dissected in cold phosphate-buffered saline (PBS) and subsequently fixed in 0.2% glutaraldehyde, 2% paraformaldehyde, 2 mM EGTA, 2 mM MgCl<sub>2</sub> in 100 mM Pipes buffer, pH 6.9, at room temperature for 30–90 min depending on the size of the embryos, then washed three times in PBS at room temperature for 20 min each. Staining was performed at 37°C in 0.02% X-Gal, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub> in PBS overnight. The stained embryos were washed, postfixed in 4% paraformaldehyde, 2% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Sections were mounted onto glass slides and counterstained with Nuclear Fast red.

Later stage embryos (~E10.5) were fixed for 2–4 h at 4°C in 4% paraformaldehyde, washed in PBS, and incubated in 15% and then 30% sucrose at 4°C overnight. Embryos were embedded in Tissue Tek (OCT compound), frozen on dry ice, and cryosectioned between 10 and 20 μm. Cryosections were washed in PBS and incubated overnight at 37°C with the same X-Gal solution as described above. Finally, the sections were washed and counterstained with eosin.

**RESULTS**

**Generation of a VEGF-LacZ Knock-in Mouse Model**

To overcome the heterozygous lethality of the VEGF null mutation, we introduced the sequence encoding the β-galactosidase reporter (LacZ cassette) into the noncoding region of exon 8, 3' of the VEGF stop codon. An IRES preceded the LacZ coding sequence. Consequently, our strategy permits the production of two functional proteins (VEGF and LacZ) from a bicistronic transcript.

The targeting vector was generated using a 6-kb Smal fragment of genomic DNA spanning exons 4 to 8 as the 5' arm of homology and a 4.4-kb Smal fragment of sequence encompassing the 3' UTR as the 3' arm of homology (Fig. 1A). The IRES-LacZ cassette followed by a loxP-flanked neomycin resistance cassette, which carried its own promoter (PGK) and polyadenylation site (also from PGK), was introduced into exon 8 after the VEGF stop codon (Fig. 1A).

After electroporation of ES cells, targeted clones were selected for neomycin resistance and homologous recombination events were identified by Southern blot analysis, with a targeting frequency of approximately 25%. Three independent targeted ES cell lines were used to generate chimeric mice by ES cell → embryo aggregation, which all transmitted the targeted allele (VEGF-LacZ-KI) through their germ line (Fig. 1B, lane +/KI). In order to minimize interference, the PGK-neo cassette was deleted by crossing the VEGF-LacZ-KI heterozygous mice with CMV-Cre transgenic mice (Nagy et al., 1998), generating the VEGF-LacZ KI→neo heterozygous mutant mice. The excision was detected by PCR (data not shown) and confirmed by Southern blot analysis (Fig. 1B, lane +/KI,neo). All lines gave identical LacZ staining, so all data were pooled.

**VEG in Early Implantation**

VEGF was first detected through LacZ staining at embryonic day E4.0 in the primitive endoderm of the blastocyst (Figs. 2B and 2C). LacZ staining was not detectable in E3.5 blastocysts (Fig. 2A). Prior to gastrulation at E5.5, LacZ was strongly expressed in the extraembryonic visceral and parietal endoderm (Fig. 2D). A lower level of expression was observed in the extraembryonic ectoderm but this signal as well as the signal in the extraembryonic parietal endoderm decreased after cavitation by E6.0 (Fig. 2E). The ectoplacental cone and the chorion, which derived from the extraembryonic ectoderm, were completely negative at E7.5 (Fig. 2G). The only trophectoderm-derived cells expressing LacZ from the VEGF locus are the primary giant cells invading the maternal decidua (arrowheads in Fig. 2D).

During the process of yolk sac vasculogenesis, there is proliferation of mesodermal cells called hemangioblasts. These cells give rise to the angioblasts, the precursors of endothelial cells, and to the precursors of hematopoietic cells (Risau and Flamme, 1995). These two cell populations form the first blood islands at around E7.0–E7.5. At this stage, VEGF is strongly expressed in the extraembryonic compartment of the embryo (Fig. 2F), but not in the embryo proper. Sections of these embryos show that LacZ expression from the VEGF locus is abundant in the extraembryonic visceral endoderm, cells characterized by
FIG. 1. VEGF-LacZ knock-in strategy and germline transmission analysis. (A) Partial restriction maps of the targeting construct, the mouse VEGF genomic fragment (WT), and the predicted structure of the targeted VEGF alleles (VEGF-LacZ-KI and VEGF-LacZ-KI\textsuperscript{\textDelta}neo). Solid rectangles represent translated exons (exon 1 to 8) and hatched boxes correspond to the untranslated regions of exons 1 and 8. The IRES-LacZ cassette was inserted into the 3' UTR of the VEGF exon 8, followed by the selectable marker PGK-neo placed between two loxP sites, indicated by arrows. The VEGF KI\textsuperscript{\textDelta}neo allele illustrates the structure of the mutant allele after the removal of the selectable marker.
a cuboidal, or columnar, shape with large numbers of vacuoles. In contrast, only a few cells of the visceral endoderm at the embryonic pole exhibit a low level of reporter expression (Fig. 2G). β-Galactosidase expression is also seen in the cells derived from extraembryonic mesoderm that form the inner layer of the yolk sac, while the adjacent hemangioblasts show no expression (Fig. 2G). The distribution of labeled cells in the allantois is

FIG. 2. VEGF expression during early embryogenesis in the extraembryonic tissues. LacZ staining of whole-mount embryos (A, B, C, F) or sections of LacZ stained embryos (D, E, G, H, I). Embryonic stages: (A) E3.5, (B) E4.0, (C) E4.5, (D) E5.5, (E) E6.5, (F, G) E7.5, (H, I) E8.0. Abbreviations: PEn, primitive endoderm; XEc, extraembryonic ectoderm; XEn, extraembryonic endoderm; V, visceral; P, parietal; XVEn, extraembryonic visceral endoderm; EEC, embryonic ectoderm; X, extraembryonic part of the embryo; E, embryonic part of the embryo; EPC, ectoplacental cone; Ch, chorion; XMe, extraembryonic mesoderm; Al, allantois; Hem, hemangioblasts; EEn, embryonic endoderm; Bls, blood islands; Bl, primary blood cells; EC, endothelial cells. Arrowheads in the panel D indicate the trophoblast giant cells. Size bar: (D, E, H, I) 25 μm, (G) 50 μm.
sparse and the nonexpressing cells may in fact represent the angioblast population (Fig. 2H). By day E8.0 the yolk sac vasculature is formed by fusion of blood islands. LacZ/VEGF expression is maintained in the extraembryonic visceral endoderm and extraembryonic mesoderm of the yolk sac, whereas the endothelium and primary blood cells are negative (Fig. 2I).

**FIG. 3. VEGF and flk-1 gene expression during embryonic vasculature development.** Whole-mount LacZ staining of Flk-1/LacZ heterozygous embryos (A, B, C) and VEGF KI (D, E, F). Sections of VEGF KI LacZ stained embryos are presented in the G, H, and I. Embryonic stages: (A, D, G) E8.0, (B, E, H) E8.5, and (C, F, I) E9.0. Abbreviations: FG, foregut; HM, head mesenchyme; NT, neural tube; GT, gut, HT, heart. Arrows in A, B, D, and E represent the dorsal aortae axis. Size bar: (G) 25 µm, (H) 50 µm, (I) 100 µm.

VEGF in the Embryo between Day E8.0 and Day E9.0

Vascularization of the embryo proper begins between E8.0 and E9.0 (Figs. 3D–3I). The first LacZ/VEGF-positive cells appear at day E8.0 in the definitive endoderm of the embryo, which forms the foregut pocket and the hindgut.
diverticulum (Figs. 3D and 3G). At this stage, the dorsal aorta is formed by vasculogenesis in close apposition to the embryonic endoderm; it is even more visible from the lateral view of the embryo (Fig. 3A) (Coffin and Poole, 1988). To visualize endothelial cells at this developmental stage we took advantage of a flk-1-targeted allele mouse line carrying a LacZ reporter gene introduced into the flk-1 locus (Shalaby et al., 1995).

At day E8.5 the primitive gut, heart, and headfolds are all positive for LacZ/VEGF expression (Fig. 3E). Sections show strong expression in the endoderm (gut epithelium), whereas expression is moderate in mesodermal derived tissues such as the heart (myocardium and endocardium) and the cephalic mesenchyme (Fig. 3H). The organ distribution of LacZ/VEGF expression correlates with the expansion of endothelial cells in the head mesenchyme and the heart as shown in Fig. 3B (lateral and frontal views of the same embryo) by expression of the LacZ/flk-1 allele.

Beginning from E9.0, LacZ/VEGF is extensively expressed in the embryo (Fig. 3F), in parallel with its vascularization as is indicated by LacZ/flk-1 expression (Fig. 3C). Notably, the expression of LacZ/VEGF is now also detectable in the neural tube (Fig. 3I).

VEGF in the Heart

Development of the heart is complex and production of a well-defined four-chamber compartment from a single tube requires a number of steps, including turning, septation, and remodeling. The primary heart tube comprises two layers, the outer myocardium and an inner endocardial layer, which it surrounds. Both of these are derived from mesodermal cells which emerge at the base of the headfold. The future myocardium forms the cardiogenic plate, and the endocardial cells proliferate subjacent to this structure. At E8.0, when the cardiogenic plate appears, LacZ/VEGF is barely detectable in the cardiac precursor cells (Fig. 4A). A few endocardial cells appear between the cardiogenic plate and the embryonic endoderm but they do not stain for LacZ (Fig. 4A). By day E8.5 the myocardium and part of the endocardium are strongly positive for LacZ staining (Fig. 4B). The endocardium is derived from two cell populations, mesodermal cells localized in the cardiogenic mesenchyme in close proximity to the endoderm and mesodermal cells migrating from the lateral plate and head mesenchyme (Eisenberg and Markwald, 1995). The formation of the endocardium by these two precursor populations might explain the differential expression of LacZ/VEGF observed in this layer. At day E9.0, LacZ/VEGF-positive endocardial cells are localized along the entire length of the heart tube, in the ventral aorta, in the aortic sac, in the bulbus cordis, and in the sinus venosus (Fig. 4C). By E9.5, LacZ/VEGF expression in endocardial cells becomes restricted to the outflow tract and the atrioventricular canal (Figs. 4D–4F). Endocardial cells lining the aortic sac as well as those that are lining the cushions show strong expression of LacZ/VEGF, whereas the endocardial cells that have undergone mesenchymal transition to form the cushion are negative (Fig. 4G). The shape of these LacZ-positive cells is round compared to the elongated shape of endothelial cells present in the yolk sac or the ventricle. LacZ/VEGF-expressing endocardial cells are located at the cushion borders of the truncus arteriosus (Fig. 4H) and in the atrioventricular canal (Fig. 4I). After the formation of the cushions and valves, LacZ/VEGF is no longer expressed in these cells but persists in the cardiac outflow tract (Fig. 4K). The endocardium of the ventricle, atrium, and even the sinus venosus is now completely negative for LacZ staining. The site of emergence of the aorta from the heart is the only location where LacZ/VEGF is detectable in vascular endothelium. Expression in the aorta declines with increasing distance from the heart. In the myocardium, compact and trabecular layers express LacZ/VEGF, and there was a higher level of expression in the septum transversum surrounding the atrioventricular canal (Figs. 4F and 4I).

At E14.5, there was very strong LacZ staining in the ventricles and a lower level of staining in the atria (Fig. 4J). The compact and trabecular myocardial layers express LacZ/VEGF at a high level and an intense blue staining is also observed in the spiral and interventricular septae (Fig. 4K). The endocardium and the endocardium-derived cushions do not express LacZ/VEGF, with the exception of the endocardial cells lining the outflow tract (Fig. 4K). Interestingly, flk-1 expression at E13.5, visualized by the LacZ knock-in (Shalaby et al., 1995) allele (LacZ/flk-1), parallels that of LacZ/VEGF in the heart, being higher in the ventricles than in the atria (Fig. 4L). However, in the ventricles, its expression is complementary to VEGF since Flk-1-positive cells are present throughout the endocardium with the exception of the endocardial cushions (Fig. 4M). In the entire embryo, the only cell population found to be expressing both VEGF and its receptor Flk-1 was the endothelium lining the outflow tract (Figs. 4K and 4M).

VEGF during Organogenesis

Sections of VEGF LacZ knock-in heterozygous embryos during the second half of gestation were examined in order to evaluate the potential role of VEGF during organogenesis. In Fig. 5A, whole-mount LacZ staining of a day E14.5 embryo indicates that LacZ/VEGF is widely expressed throughout the embryo. However, sections revealed that expression is restricted, with strong staining in particular organs and no staining in others (Figs. 5B and 5C).

Most organs are vascularized by either vasculogenesis or angiogenesis. Chimeric analyses have shown that in organs derived from mesoderm and endoderm vascularization occurs primarily through vasculogenesis while organs derived from ectoderm–mesoderm are vascularized by angiogenesis (Pardanaud et al., 1989). The levels of VEGF expression appear to relate to these two mechanisms. In mesoderm- or endoderm-derived tissues, like the lung and spleen, LacZ/
VEGF staining is very strong and uniform (Figs. 5H and 5J). The vasculature of these organs, detected by LacZ/flk-1, is dense and perfectly complements VEGF-expressing cells (Figs. 5I and 5K).

The brain is vascularized by angiogenesis. Here, LacZ/VEGF expression is moderate and present mainly in the ventral layer of the neuroepithelium (Fig. 5F). Angiogenesis is very active in this tissue and LacZ/flk-1 staining shows
that the newly formed blood vessels penetrated the neuroepithelium perpendicularly and grew forward towards the highly VEGF-positive ventral layer (Fig. 5G). In the kidney, LacZ/VEGF-expressing cells are restricted to the epithelium of the ureteric bud, the surrounding mesenchyme and the podocytes, with almost no expression in the outer cortex (Fig. 5D). Vascularization of the kidney proceeds partially by angiogenesis, and LacZ/flk-1 is highly expressed in vessels surrounding the ureteric bud and not at the periphery of the kidney (Fig. 5E).

**VEGF Close to Fenestrated Endothelium**

During organogenesis, the endothelium undergoes maturation and specialization to match the needs of each particular organ function. Microvascular endothelium can be
subdivided into continuous and discontinuous endothelium. Fenestrated endothelium represents a specialized subtype of discontinuous endothelium and is characterized by small pores covered by diaphragms (Bennett et al., 1959; Risau, 1995). Fenestrated endothelium is found in close proximity to epithelia that have a functional requirement for permeable endothelium. Figures 6A–6D show the pattern of expression of VEGF in organs in which fenestrated endothelium is present. In endocrine glands, like the adrenal gland, which sustain a secretory role, LacZ/VEGF is abundant (Fig. 6A). The same is true for the epithelial cells of the choroid plexus (Fig. 6B). In the kidney, the glomerular podocytes play the role of a filtration unit, and LacZ/VEGF is also present in these cells (Fig. 6C). Fenestrated endothelium is also described in the gastrointestinal tract in association with its absorptive function and LacZ/VEGF is localized in the stomach and intestine (Figs. 5B and 6D). In comparison, LacZ/flk-1 is present in the same tissues with a high level of expression in all endothelial cells that are situated in close contact to the different VEGF-positive epithelium (Figs. 6E–6H).

**FIG. 6.** VEGF expression in developing organs containing fenestrated endothelium (A–D) or during secondary inductive interactions (I–L). Flk-1 expression in fenestrated endothelium (E–H). Sections of different organs of day E14.5 VEGF-KI or E13.5 Flk-1KO LacZ-stained embryos. (A, E) Adrenal gland, (B, F) choroid plexus, (C, G) kidney (PO, podocytes; G, glomerula), (D, H) stomach, (I) whisker follicles (P, dermal papilla; E, epidermal hair bulb), (J) mammary gland (DM, dense mesenchyme; E, mammary epithelium), (K) pituitary gland (N, neurohypophysis; l, pars intermediate; D, pars distalis), (L) tooth primordium (S, mesenchymal dental sac; P, dental papilla; L, dental lamina). Size bar: (A–J) 25 μm, (K, L) 50 μm.

**VEGF during Inductive Tissue Interactions**

Inductive tissue interactions constitute a general mechanism that is observed throughout embryonic development. During organogenesis, secondary inductive events occur between epithelial and mesenchymal tissues, leading to the organization of cells into tissues or organs (Gilbert, 1988). LacZ/VEGF expression was examined during the formation of organs dependent on secondary induction events and it was usually spatially restricted in these tissues. For in-
stance, in the whisker follicle, LacZ/VEGF was expressed in the dermal papilla, which is derived from condensed mesenchymal cells developing under the skin surface, but not in the hair bulb, which arises from epidermal structures (Fig. 6I). In contrast, during mammary gland development, LacZ/VEGF was present in the ectoderm-derived epithelium but not in the dense mammary mesenchyme surrounding the embryonic mammary bud (Fig. 6J). The pituitary gland is formed by the interaction of two ectodermal derivatives, Rathke’s pouch, which is derived from the oral ectoderm, and a neuroectodermal invagination. At E14.5, LacZ/VEGF was found in the neurohypophysis and at a lower level in the pars distalis, which proceed to form the posterior and the anterior lobes of the hypophysis, respectively (Fig. 6K). The pars intermediate does not express LacZ/VEGF (Fig. 6K). During tooth development, LacZ/VEGF is located in the dental lamina, which is derived from the oral ectoderm, and is especially high in the enamel knot in the dental epithelium. The neural-crest-derived dental papilla does not express LacZ/VEGF, whereas the mesenchymal dental sac that surrounds the tooth germ does (Fig. 6L). The pattern of expression of LacZ/VEGF during organogenesis is well-defined, as it is in the early embryo, which suggests a role for VEGF during the entire process of embryonic development.

**VEGF in the Female Reproductive Tract**

To validate the use of this mouse model to study VEGF expression during physiopathological processes, we looked at the LacZ/VEGF expression during the vascular remodeling that occurs during the ovarian cycle and embryonic implantation. VEGF expression during those processes has already been characterized (Ferrara et al., 1998; Maisonpierre et al., 1997; Shweiki et al., 1993). We confirmed by LacZ staining that VEGF is present in the preovulatory follicle before vessel invasion and is first detectable in cells of the granulosa that surround the oocyte (Figs. 7A–7D). After ovulation, LacZ/VEGF is strongly expressed in the developing corpus luteum (Figs. 7E–7H). At E7.0–9.0, when angiogenesis is occurring in the maternal decidua and newly formed blood vessels are converging on the embryo, VEGF and LacZ/VEGF is expressed in these cells (Figs. 7I and 7J). Decidual cells express high level of LacZ/VEGF with the exception of the deepest layer of the endometrium (Figs. 7K and 7L). The same profile is observed by in situ hybridization (Shweiki et al., 1993). The myometrium remains negative for LacZ/VEGF staining (Fig. 7L).

**DISCUSSION**

The pivotal role that VEGF plays in normal development and disease has been revealed by gene targeting experiments (Carmeliet et al., 1996; Ferrara et al., 1996). To understand the details of this role we have created a new VEGF allele by homologous recombination. This allele allows independent translation of VEGF and LacZ from the same bicistronic mRNA. Not only did this approach yield a convenient LacZ tag for VEGF expression but it also circumvented the lethal haploinsufficiency of VEGF. This strategy presents several advantages over in situ hybridization. β-Galactosidase activity is both easily detectable and very sensitive. Moreover, reporter activity can be detected at single cell level thus providing the opportunity to clearly determine the identity of the cells expressing VEGF. The IRES element translating LacZ from the 3’ UTR of VEGF did not appear to compromise VEGF translation from the bicistronic mRNA produced. Therefore, the heterozygous mice carrying the VEGF-LacZ KI are viable and provide an invaluable tool for characterizing VEGF expression. This resolution gave a unique opportunity to pinpoint the detailed roles of VEGF during development. Some of these have been already indicated by experiments describing VEGF expression using the less sensitive RNA in situ hybridization (Breier et al., 1992; Dumont et al., 1995; Peters et al., 1993).

In this study, we report that embryonic expression of VEGF initiates in the primitive endoderm of E4.0 blastocysts, a time when VEGF mRNA is also detectable by RT-PCR (data not shown). As gastrulation proceeds, a common progenitor to hematopoietic and vascular cells, called the hemangioblast, is believed to arise from the mesoderm located at the posterior primitive streak and to colonize the yolk sac (Dieterlen-Liévre, 1998). Mouse embryonic explant experiments recently demonstrated that signals from the primitive endoderm are required around the onset of gastrulation for hematopoietic and vascular development (Belauossoff et al., 1998). VEGF expression in the extraembryonic visceral endoderm of the yolk sac may provide the paracrine signal essential for the differentiation of endothelial cells from angioblasts (Risau and Flamme, 1995). In agreement with this suggested role, VEGFIR2/Flik-1-deficient embryos failed to produce mature endothelial cells; however, immature angioblasts were able to form (Shalaby et al., 1995). Confirming previous results of in situ hybridization (Dumont et al., 1995), LacZ/VEGF is highly expressed in the extraembryonic visceral endoderm during vasculogenesis. With our system we were able to extend these observations by showing that LacZ expression is also detectable in the mesodermal component of the yolk sac. However, LacZ/VEGF is not detectable in either the blood islands or the mature endothelial cells of the yolk sac. This expression pattern strongly supports the earlier suggestion that VEGF may act as a paracrine signal regulating vasculogenesis (Risau and Flamme, 1995), by inducing differentiation of the angioblasts and proliferation and migration of the arising endothelial cells in the yolk sac in order to form a primary vascular network.

It is believed that the endothelial cells of the embryo proper arise from two types of progenitors (Pardanaud et al., 1996). The first type of progenitor is purely angioblastic and
derives from the somitic mesoderm, whereas the second type is localized at the paraaortic splanchnopleura and shares common properties with the yolk sac hemangio- blasts (Godin et al., 1993; Jaffredo et al., 1998). In the embryo proper expression of VEGF begins in an endodermal lineage since it is strongly expressed in both the foregut and the hindgut. The dorsal aorta, the first vessel formed in the embryo proper, arises by vasculogenesis with the contribution of both types of precursors (Coffin and Poole, 1988; Poole and Coffin, 1989). This process proceeds in close proximity to the primitive gut, a region exhibiting high LacZ/VEGF expression. The VEGF knockout has shown that partial or complete lack of VEGF impairs the formation of the dorsal aorta (Carmeliet et al., 1996; Ferrara et al., 1996). In avian embryos, quail–chick transplantations demonstrate that the endoderm induces angioblastic and hematopoietic development (Pardanaud and Dieterlen-Liévre, 1999). Moreover, this hemangioblastic induction can be mimicked by VEGF and also by bFGF and TGFβ. Taken together, these findings suggest that the endodermal compartment plays an essential role in the formation of the dorsal aorta by providing VEGF as the paracrine signal for their development. This appears to be true across species. In zebrafish, a signal from the notochord plays an important role in the formation of the dorsal aorta, but VEGF localization has not been determined in this organism (Fouquet et al., 1997; Sumoy et al., 1997). Similarly, in Xenopus, a signal from the hypochord is required for the formation of

**FIG. 7.** VEGF expression in the female reproductive tract. (A–H) VEGF expression during vascular remodeling in the ovarian cycle. Preovulatory stages are presented by whole-mount LacZ staining of the entire ovary (A) or a detail of a preovulatory follicle (B) and by sections showing a small follicle (C) and a large preovulatory follicle (D). Postovulatory stages are presented by whole-mount LacZ staining of the entire ovary (E) or a detail of a corpus luteum (F). Sections of this ovary show LacZ/VEGF staining in a developing corpus luteum –8 h (G) or –12 h (H) after ovulation. (I–L) VEGF expression in the decidua and embryonic implantation site. Whole-mount LacZ staining of decidual tissue of E7.5 (I) and E9.5 (J) embryo. Sections of an E7.5 embryo surrounded by the maternal decidua and uterine muscle wall (K, L). Abbreviations: O, oocyte; G, granulosa; CL, corpus luteum; E, embryo; M, maternal decidua; U, uterus; EN, endometrium; M, muscle layer of the uterine wall. Size bar: (C) 25 μm, (D, G, H, L) 50 μm, (K) 100 μm.
the aorta (Cleaver et al., 1997). This structure, absent in other vertebrates, is induced by the notochord itself and produces VEGF, which mediates angioblast migration during the dorsal aorta formation (Cleaver and Krieg, 1998). These findings indicate that it is not the embryonic endoderm per se but rather a factor such as VEGF secreted by this germ layer that is required for a proper formation of the dorsal aorta.

At E8.5, LacZ/VEGF is also detectable in the head mesenchyme, which contains a second pool of pure angioblasts. In chick embryos, the angiogenic potential of the cephalic mesenchyme has been demonstrated by quail/chick transplantations (Couly et al., 1995). This angiogenic potential could be mimicked by direct contact between mesoderm and ectoderm or by EGF/TGFβ treatments of the mesoderm (Pardanaud and Dieterlen-Liévre, 1999). Nevertheless, our findings suggest that the angiogenic potential of the head mesenchyme may also be associated with VEGF expression, though this expression is relatively low compared to the expression observed in the endoderm layers. Angioblasts were found in the head mesenchyme of the VEGF and Flk-1 knockout embryos but they were of an immature phenotype and could not form proper vessels (Carmeliet et al., 1996; Shalaby et al., 1995). These results as well as those obtained in avian embryos suggest that VEGF is probably not the only factor to induce this angioblastic population, but appears to play a role in the differentiation and recruitment of these cells to form an ordered vascular network.

In addition to having a crucial role in establishing embryonic vasculature, VEGF may be functionally involved in organogenesis. We demonstrate that VEGF is expressed in the heart, starting at a low level around E8.5 in the cardiogenic plate and increasing as the heart develops. We observed a stronger expression in the ventricles than in the atria and elevated expression in the septa and trabeculae compared to the expression in the compact ventricle wall. During cardiac development, the close apposition of two cell populations, the myocardium and the endocardium, appears to be necessary to coordinate the proper development of the heart. Endocardial cells arise from the mesoderm and form a single cell layer between the cardiogenic plate and the endoderm, and it has been shown by explant cultures that the endoderm plays a role in directing endocardial development (Sugi and Markwald, 1996). VEGF is present at a high level in the endodermal cell layer and could represent one of the signals responsible for the development of the endocardium. This hypothesis is supported by an absence of endocardial cells in the hearts of the Flk-1-deficient embryos (Shalaby et al., 1995). Endocardial/myocardial interactions are also important for generating trabeculation in the ventricles (review Fishman and Chien, 1997) and the endocardium is crucial for the formation of the heart valves and cushions. In vitro studies have suggested that the mesenchymal transition of the endocardium to form the cushions is under the control of a localized myocardial signal. When we consider these observations in light of the LacZ/VEGF cardiac expression, we note a high level of VEGF in the myocardial cells that are in close contact to the endocardium. This association suggests that VEGF could be involved in the interaction between endocardium and myocardium. In addition, data demonstrating that an elevated level of VEGF in the developing heart can induce heart failure in the mouse (L. Miqueel et al., in preparation) and in the quail (Feucht et al., 1997) strongly support this hypothesis.

Targeted disruption of VEGF has demonstrated that the level of VEGF expression is of major importance for normal embryonic development. However, because of the early lethality of the VEGF−/− and VEGF+/− embryos, observations are restricted to stages at which vasculogenesis predominates. Endoderm/mesoderm-derived tissues that are believed to be vascularized by vasculogenesis show a higher and a homogeneous pattern of expression of LacZ/VEGF compared to ectoderm/mesoderm-derived tissues vascularized by angiogenic processes. In particular, a high level of LacZ/VEGF expression is found in the yolk sac and gut endoderm where vasculogenesis leads to the formation of blood islands and dorsal aortae, respectively. During organogenesis, the reporter is highly expressed in spleen and lung, tissues that are thought to be vascularized by vasculogenesis (Coffin and Poole, 1988; Pardanaud et al., 1989). In contrast expression is low in the brain and neuroectoderm, examples of tissues in which the vascular plexus is formed by angiogenesis.

Vascularization of the kidney is complex and probably involves both vasculogenesis and angiogenesis (Abrahamson et al., 1998). In the kidney, VEGF is expressed at different levels and in several different types of cells and suggests us a correlation between the expression level and the function. VEGF is expressed in the ureteric bud epithelium and the surrounding mesenchyme. Similar analyses using LacZ/flk-1 mice revealed that the first Flk-1-positive cells arise along the ureteric bud (Robert et al., 1998). The localization of VEGF may serve as a source of chemotactic protein for the outside vasculature that colonized the kidney. Very high VEGF expression is detected in the podocytes, which form the epithelium of the glomeruli, whereas Flk-1 is localized to the glomerular endothelium. In the glomeruli, where the endothelium is fenestrated, this permeable endothelium is always located in close proximity to the epithelium. Fenestrated endothelium has also been shown to be present in tumors in which VEGF is induced (Senger et al., 1993). Given its documented permeability-inducing activity, VEGF appears to be a candidate for the induction and the maintenance of the fenestrated capillary phenotype (Esser et al., 1998; Risau, 1998).

Our data confirm the complementary expression of VEGF and flk-1 during embryonic development (Dumont et al., 1995; Flamme et al., 1995; Millauer et al., 1993). These genes are exclusively expressed in two different cell types;
Flk-1 is expressed in the endothelium and VEGF in the neighboring cells. With the VEGF LacZ-KI model, we have shown that one subcompartment of the endocardium, endothelial-like cells, is able to produce both VEGF and Flk-1. It is worth noting that some of these cells are predestined to undergo mesenchymal transition in order to form the cardiac cushions. Thus, these cells do not present a strict vascular phenotype like the endothelial cells that line the vasculature and might account for their VEGF expression in these cells. This is not true for the endothelium, which lines the aorta of the outflow tract and also expresses VEGF. Thus, VEGF expression is not entirely excluded from the endothelial cells as was originally assumed (Risau and Flamme, 1995; Shifren et al., 1994). In quail embryo, VEGF expression is detected in the endothelium of the aorta close to the heart, suggesting a potential autocrine regulation (Aitkenhead et al., 1998).

In summary, LacZ tagging of the VEGF allele has allowed precise analysis of the pattern of VEGF expression at a cellular level. This detailed characterization has revealed important information that strongly suggests multiple functions for VEGF during embryonic development. This model can provide a convenient readout for VEGF expression in normal physiological processes as well as in pathological situations.

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