HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Identification of Vascular Endothelial Growth Factor (VEGF) Receptor-2 (Flk-1) Promoter/Enhancer Sequences Sufficient for Angioblast and Endothelial Cell-Specific Transcription in Transgenic Mice

By Andreas Kappel, Volker Rönicke, Annette Damert, Ingo Flamme, Werner Risau,† and Georg Breier

The vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) is the first endothelial receptor tyrosine kinase to be expressed in angioblast precursors, and its function is essential for the differentiation of endothelial cells and hematopoietic precursors. We have identified cis-acting regulatory elements of the murine Flk-1 gene that mediate endothelium-specific expression of a LacZ reporter gene in transgenic mice. Sequences within the 5′-flanking region of the Flk-1 gene, in combination with sequences located in the first intron, specifically targeted transgene expression to angioblasts and endothelial cells of transgenic mice. The intronic regulatory sequences functioned as an autonomous endothelium-specific enhancer. Sequences of the 5′-flanking region contributed to a strong, uniform, and reproducible transgene expression and were stimulated by the transcription factor HIF-2α. The Flk-1 gene regulatory elements described in this study should allow the elucidation of the molecular mechanisms involved in endothelial cell differentiation and angiogenesis.

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THE CARDIOVASCULAR SYSTEM IS THE first functional organ system to be formed in the vertebrate embryo. Endothelial cells, which constitute the inner lining of all blood vessels, differentiate from mesodermal precursor cells, or angioblasts, shortly after gastrulation.1-2 The close association of angioblasts and hematopoietic precursor cells at this developmental stage is thought to reflect their origin from a putative common precursor cell, the hemangioblast. In a process termed vasculogenesis, angioblasts aggregate to form a primitive vascular plexus, and differentiate into endothelial cells. The primitive vascular plexus is then further refined by angiogenesis and remodeling.

Angiogenic growth factors of the vascular endothelial growth factor (VEGF) family and their cognate endothelial receptors, Flt-1 (VEGF receptor-1), Flk-1/KDR (VEGF receptor-2), and Flt-4 (VEGF receptor-3) function as signaling molecules during vascular development.3 Gene targeting experiments have shown that these molecules have an essential function in embryonic vascular development.4 VEGF and the VEGF receptors represent the first endothelial cell-specific signal transduction pathway known to be activated during vascular development.5-8 In particular, Flk-1 appears to play a pivotal role in endothelial cell differentiation and vasculogenesis. Flk-1 is the first endothelial receptor known to be expressed in the primitive mesoderm,9,10 and mice that are homozygously defective for Flk-1 completely lack mature endothelial cells and blood vessels.6,11 Moreover, hematopoiesis is defective in the Flk-1 −/− mice, indicating that Flk-1 function is also essential for the differentiation of hematopoietic precursors. This is consistent with the hypothesis that Flk-1 is a marker for the hemangioblast.12 Collectively, these data provide evidence that Flk-1 gene activation is a principal event leading to the emergence of the hemangioblastic lineage and the differentiation of endothelial and hematopoietic cells. During later stages of embryonic development Flk-1 is highly expressed on endothelial cells,9,13 but is downregulated in most hematopoietic cells.14 In the adult, Flk-1 expression is not detectable in most vascular beds. The strong induction of Flk-1 expression in tumoral endothelial cells is involved in the neovascularization of various human or experimental tumors.15,16 Thus, the understanding of Flk-1 gene regulation represents a key step in the understanding of the mechanisms involved in endothelial lineage differentiation and angiogenesis in development and disease. The identification of the cis-acting elements in the Flk-1 gene that are responsible for its unique expression profile provides a valuable tool for the identification of upstream factors that control the establishment of the hemangioblastic lineage.

We have previously isolated genomic clones that encompass the promoter region of the murine Flk-1 gene, and have performed a functional analysis of the Flk-1 promoter in vitro.17 We17 and others18 showed that Flk-1 5′-flanking sequences confer endothelium-specific expression in transfected endothelial cells. The significance of these cis-acting elements for the developmental expression of the Flk-1 gene in vivo remained unclear. In this report, we have characterized the murine Flk-1 regulatory sequences in transgenic mice. Despite their activity in cultured endothelial cells, 5′-flanking sequences alone could not target expression of a LacZ reporter gene to the endothelium of mouse embryos. However, in combination with sequences from the first intron of the Flk-1 gene, the Flk-1 promoter could specifically drive reporter gene expression in endothelial cells. The transgene expression pattern closely resembled that of the endogenous Flk-1 gene throughout development. The intron sequences conferred endothelium-specific gene expression also to the heterologous herpes simplex virus-thymidine kinase (tk) promoter and fulfilled all the criteria of an autonomous tissuespecific enhancer. The Flk-1 promoter sequences were essential for a strong and reproducible transgene expression, and were...
stimulated by HIF-2α, a basic helix-loop-helix/PAS domain transcription factor that is prominently expressed in the vasculature of embryonic mice.\textsuperscript{19,21}

**MATERIALS AND METHODS**

*DNA sequence analysis.* Restriction fragments of a 12 kb region of the *Flk-1* gene ranging from \(-6.5\) kb to \(+5.5\) kb relative to the transcription initiation site were subcloned into the pBluescriptII vector (Stratagene, La Jolla, CA). The nucleotide sequence was determined on both strands by using the deoxynucleotide chain termination method on an Applied Biosystems 373 automated sequencer. The nucleotide sequence of the *Flk-1* intron enhancer is deposited in the Genbank database (accession number AF061804). The search for potential transcription factor binding sites was performed with the MatInspector software (GBF, Braunschweig, Germany).\textsuperscript{22}

*Plasmid construction and transient transfection.* The LacZ reporter vector was generated by inserting the LacZ cassette into the HindIII and BamHI restriction sites of the pGL2-basic plasmid (Promega, Madison, WI), thereby exchanging the luciferase gene against the LacZ cassette. The LacZ cassette was derived from the pBacinSDKLacZ plasmid (a gift from Janet Rossant, Samuel-Lunenfeld-Research Institute, Toronto, Canada). *Flk-1* promoter fragments were amplified by the polymerase chain reaction (PCR) as described\textsuperscript{17} and inserted into the KpnI and HindIII sites of pGL2. The \(-4.1\) kb \(+299\) bp fragment was generated by inserting a *Flk-1* promoter fragment ranging from \(-4.1\) kb to \(-1.9\) kb into the HindIII and EcoRI sites of the pGL2-Basic plasmid that already contained the *Flk-1* promoter region from \(-1.9\) kb to \(+299\) bp, and subsequently exchanging the luciferase gene against the LacZ cassette. The \(-640\) bp \(+299\) bp promoter fragment was amplified as described\textsuperscript{27} using the forward primer Flk-640: Flk-640: 5'-GGGG-TACCTTCTGGACCGACCCAAGCGAAG-3'.

*Flk-1 intron fragments were amplified by PCR as described.\textsuperscript{17}* PCR products were digested with BamH1 and XhoI or BamH1 and SalI and inserted into the BamH1 and SalI restriction sites downstream of the LacZ cassette in the modified pGL2-Basic vector that already contained the *Flk-1* promoter fragment. A recombinant lambda phage clone, P16\textsuperscript{27} served as a template for PCR amplification. The following primers were used: 5'-In1fw: 5'-AGGGATCCACTCCTTTTAGTAGTAAGGCG-3', 5'-In1rev: 5'-ACCTCGAGATCTGATTGCCAC-3', 3'-In1fw: 5'-GGGTATATGCTGTGTTCCC-3', 3'-In1rev: 5'-GGATGGGGAA-AATCCGCGCCG-3', In2fw: 5'-GTTGCTGATTTTGTTAGGAGG-3', In2rev: 5'-CATAGAGGAAACAAGCTGGGAGG-3'. The 510 bp intron fragment located between nucleotides +3437 and +3947 was a SmaI/BamHI fragment derived from recombinant phage P16\textsuperscript{17} and was inserted by blunt-end cloning to the blunt *BamHI* site of the modified pGL2.

*Bovine aortic endothelial (BAE) cells, NIH 3T3 cells, and A293 cells were isolated from a mouse brain capillary cDNA library\textsuperscript{23} using the IHI fragment derived from recombinant phage P16.\textsuperscript{17} The 510 bp fragment was inserted into the SalI restriction sites of the pGL2-Basic plasmid. Then, the luciferase reporter vector was constructed by inserting the lacZ cassette into the EcorI and NotI sites of pcDNA3 (Invitrogen, Carlsbad, CA). For cotransfection assays, A293 cells were split 1:2 into 35-mm dishes and transfected 18 hours later with 4 ng of DNA/µg of *Flk-1* promoter-driven luciferase plasmid, 1 µg of CMV promoter-driven β-galactosidase expression plasmid, and 1 µg of the HIF-1α or HIF-2α expression plasmids, or pBluescript SKII and pcDNA3 plasmids as a control) using a transfection kit (MBS, Stratagene). After 20 hours, reporter gene activity was measured using the Dual Light Kit (Tropix, Bedford, MA). The luciferase activity of each extract was normalized to the respective β-galactosidase activity. Endogenous background levels of both enzyme activities were determined from extracts from mock-transfected cells and were subtracted. The normalized luciferase activity of the control transfection was arbitrarily set to 1. Each value represents the average of at least six experiments.

*Generation and analysis of transgenic mice.* Transgenic mice were generated by microinjection of fertilized mouse oocytes as described.\textsuperscript{24} Fertilized oocytes were isolated from superovulated C57BL/6 \(\times\) C3H/He F1 mice, microinjected, and reimplanted into pseudopregnant females of the same hybrid-mouse strain. Mouse embryos were analyzed by whole mount LacZ staining for transgene expression as described.\textsuperscript{25} Genomic DNA was prepared from unstained embryos or yolk sacs, and genotyping was performed by PCR analysis as described\textsuperscript{26} using the primer pair LacZP1/LacZP2. LacZP1: 5'-ATCCCT-TGATGTTGAGGGTTGTCG-3', LacZP2: 5'-CTGGATGAGGTTTGTCC-3'. For histological analysis, embryos were embedded in paraffin, and 10 µm sections were prepared and counterstained with neutral red.

Cryostat sectioning and LacZ staining of organs from postnatal mice were performed as described.\textsuperscript{26} Immunofluorescence detection of platelet endothelial cell adhesion molecule-1 (PECAM-1) was performed as described\textsuperscript{27} using a CY3-conjugated Goat antirat IgG secondary antibody (Jackson Immuno Research Laboratories Inc, West Grove, PA) as recommended by the manufacturer.

**RESULTS**

*Functional analysis of the Flk-1 promoter in transgenic mouse embryos.* We have previously characterized promoter fragments from the murine *Flk-1* gene that confer endothelium-specific expression to the firefly luciferase reporter gene in transfected BAE cells.\textsuperscript{17} Although the *Flk-1* promoter sequences extending to \(-4.1\) kb had the highest cell-type specificity of all fragments tested in vitro, a stronger promoter activity was observed with shorter promoter fragments.\textsuperscript{17} To investigate whether the *Flk-1* gene regulatory regions identified in vitro are also functional in vivo, we generated transgenic mouse embryos carrying a transgene that consists of the LacZ reporter gene under the control of different *Flk-1* promoter fragments. Analysis of transgenic embryos was performed at day 10.5 of embryonic development (E10.5). A weak vascular transgene expression was observed in 1 out of 31 transgenic embryos tested that contained a promoter fragment spanning the *Flk-1* gene from \(-1.9\) kb to \(+299\) bp relative to the transcription initiation site (Table 1). No endothelium-specific expression was observed with longer \((-4.1\) bp\/+299 bp) or shorter fragments \((-640\) bp\/+299 bp) (Table 1). Therefore, none of the various *Flk-1* promoter fragments mediated a reproducible endothelium-specific reporter gene expression in vivo. From these data we concluded that additional sequences of the *Flk-1* gene are required to confer the endogenous expression pattern.
Identification of endothelium-specific regulatory elements in the first intron of the Flk-1 gene in vitro. Many tissue-specific gene regulatory elements are located within intronic sequences. To examine whether the first or the second intron of the Flk-1 gene contains sequences that might be able to supplement the function of the Flk-1 promoter in a cell-type–specific manner, we performed reporter gene analysis in transfected cells. Several subfragments of the first two introns (5′-In1, 3′-In1, In2; shown in Fig 1A) were included in β-galactosidase reporter gene constructs together with a 4.4 kbp Flk-1 promoter fragment (−4.1 kbp/+299 bp), and transient transfection was performed in BAE and NIH 3T3 cells. For both cell types, the promoter activity of the reporter gene construct that contained the 5′ region of the first intron (5′-In1) fragment was arbitrarily set to 100 relative light units (RLU). The substitution of this intronic fragment with the 3′ region of the first intron (3′-In1), a 2.3 kbp XhoI/BamHI fragment (−1677 bp/+3947 bp), induced a twofold increase in promoter activity in BAE cells, but not in NIH 3T3 cells (Fig 1B). The second intron (In2), in contrast, did not alter the expression levels significantly (Fig 1B). These results indicate that a positive-acting endothelium-specific element is located in the 2.3 kbp XhoI/BamHI fragment of the first Flk-1 intron.

Endothelium-specific expression mediated by Flk-1 regulatory sequences in vivo. When the 2.3 kbp XhoI/BamHI fragment of the first intron (3′-In1; +1677 bp/+3947 bp; see above) was tested in combination with the Flk-1 promoter fragment (−640 bp/+299 bp), a reproducible vascular LacZ expression in transgenic E10.5 mouse embryos was observed (Table 1), for example in blood vessels of the head region, in intersomatic vessels, the dorsal aorta, and in the heart anlage (Fig 2A). Sectioning of these embryos confirmed that the β-galactosidase expression was confined to vascular endothelium (data not shown). The intron fragment could also direct endothelium-specific LacZ expression when used in an inverted orientation in the reporter construct (−640 bp/+299 bp/+3947 bp/+1677 bp, Table 1).

Transgenic mouse lines were generated with this reporter gene construct (−640 bp/+299 bp/+1677 bp/+3947 bp) containing the Flk-1 regulatory sequences. Three independent lines were obtained that showed a uniform vascular expression of the reporter gene in the embryo proper and the yolk sac at E11.5, as assessed by whole-mount LacZ staining. One of these lines (2603) was analyzed further (Fig 2B). Sectioning of β-galactosidase–stained E11.5 transgenic embryos showed that reporter gene expression was confined to the endothelium of blood vessels, eg, in the dorsal aorta (Fig 2D), in venous vessels (Fig 2E), in the perineural vascular plexus, and in capillary sprouts invading the neural tube (Fig 2F). The LacZ expression in this line colocalized with the expression of the endothelial marker PECAM1, as confirmed by the parallel immunofluorescence staining with an anti-PECAM1 antibody of adjacent sections (Fig 2G,H). The LacZ staining pattern of these embryos was also compared with heterozygous Flk-1 mutant mouse embryos that express the LacZ gene from the endogenous Flk-1 locus.6 The LacZ staining pattern of transgenic embryos and the knock-in embryos at E11.5 were indistinguishable (Fig 2B,C). These observations showed that the intron sequences in combination with the Flk-1 promoter confer an

Table 1. Summary of the In Vivo Activity of Different Flk-1 Constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>TG</th>
<th>ES</th>
<th>ET</th>
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<td>1</td>
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<tr>
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<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>7</td>
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<td>0</td>
<td>2</td>
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<tr>
<td>−640/+3947 // 3′Intron +3437/+1677</td>
<td>13</td>
<td>5</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
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<td>3</td>
<td>0</td>
<td>12</td>
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Embryos transgenic for the constructs given above were generated, and LacZ staining and genotyping were performed at E10.5 or E11.5 as described in the Materials and Methods section. Constructs are defined by the position of the promoter or intron fragments in bp relative to the transcription initiation site of the endogenous Flk-1 gene.

Abbreviations: TG, number of transgenic embryos; ES, number of embryos showing endothelial-specific staining; ET, number of embryos showing ectopic staining; NO, number of embryos showing no staining at all.
endothelium-specific expression pattern that closely resembles the expression pattern of the endogenous Flk-1 gene.\textsuperscript{13,28}

The first intron of the Flk-1 gene contains an autonomous endothelium-specific enhancer. To further assess the function of the first Flk-1 intron in endothelium-specific gene expression, we investigated whether the intron sequences can confer endothelium-specific expression to the heterologous tk promoter. This promoter has no intrinsic endothelial cell specificity.\textsuperscript{26} A LacZ reporter gene construct was generated that contained the tk promoter, in combination with the 2.3 kbp XhoI/BamHI fragment of the Flk-1 gene.\textsuperscript{26} Transgenic mouse embryos generated with this construct showed vascular reporter gene expression (Fig 2I). Based on microscopic inspection of whole mount-stained embryos, the β-galactosidase staining observed in these embryos was weaker than in embryos expressing LacZ under the control of the −640 bp/+299 bp Flk-1 promoter in combination with the intron fragment (Fig 2A,B). Moreover, the frequency of transgenic
mouse embryos expressing this transgene was significantly reduced, when compared with constructs containing the −640 bp/+299 bp Flk-1 promoter in combination with the intron fragment (Table 1). This indicates that the tk promoter lacks positive-acting elements that are present within the Flk-1 promoter. The Flk-1 intron fragment alone, in contrast to the Flk-1 promoter, can reproducibly target reporter gene expression to the endothelium and acts as an autonomous endothelium-specific enhancer.

To further characterize the minimal intron sequences that are required for endothelium-specific expression, we analyzed whether shorter intron fragments were also active in combination with the 939 bp promoter region of the Flk-1 gene (−640 bp/+299 bp). By deletion analysis, the intron enhancer was localized to a 510 bp fragment (+3437 bp/+3947 bp) that is located immediately upstream of the second exon. This fragment was sufficient to drive endothelium-specific LacZ expression in transgenic mouse embryos when tested in combination with the Flk-1 promoter (Fig 2J, Table 1). The DNA sequence of this fragment (Fig 3) contains potential binding sites for the GATA and Ets transcription factors, and for Scl/Tal-1, all of which have been proposed to play a role in angiogenesis.29-33

Flk-1 regulatory sequences target endothelium-specific transgene expression throughout development. To test whether the regulatory sequences of the Flk-1 promoter and enhancer identified can reproduce the endogenous Flk-1 expression pattern throughout development, the LacZ expression pattern of the transgenic mouse line 2603 (Fig 2B) was further analyzed at various stages of embryonic development, at postnatal day 5 (P5) and in the adult (P120). The earliest stage during which transgene expression was examined by whole-mount LacZ staining was at E7.8 (Fig 4A). The analysis of sections of these embryos confirmed that the transgene was expressed in angioblasts of the allantois and the yolk sac (Fig 4B,C). Moreover, transgene expression was restricted to the vascular endothelium at all stages of embryonic development examined (E7.8 to E14.5, data not shown).

LacZ staining was detected in vessels of the spleen, kidney, thymus, liver, and lung from P5 animals (Fig 4D to H). However, LacZ expression was downregulated in most vascular beds of adult animals, except for the spleen (data not shown). These results further support the conclusion that the identified Flk-1 regulatory sequences are sufficient to reproduce most properties of the endogenous Flk-1 expression.

The 5′-UTR of Flk-1 is required for expression in the yolk sac vasculature. In Flk-1/LacZ knock-in embryos, the LacZ gene is under control of all endogenous regulatory elements except for the regions from bp +137 to bp +299 in the 5′-UTR and approximately the first 600 bp of the first intron.8 In this study we have shown that these intron sequences are not required to generate the strong and uniform endothelial-specific reporter gene expression (Fig 2B and Table 1). The uniform vascular LacZ expression in the transgenic yolk sacs (Fig 5A, B) was absent in small vessels of the yolk sacs of the Flk-1/LacZ knock-in embryos (Fig 5C), in which only large yolk sac vessels were stained. This indicates that the region from bp +137 to bp +299 of the Flk-1 5′-UTR is required for uniform Flk-1 expression in yolk sac vessels. To verify this hypothesis, we further analyzed the effect of this deletion in transgenic mice. Deletion of the 5′-UTR sequences from bp +137 to bp +299 in the transgene construct analyzed in Fig 5A resulted in a diminished and incomplete LacZ expression in the yolk sacs of all five transgenic mouse lines tested (Fig 5D), and in a reduced frequency of transgenic embryos expressing LacZ (Table 1, construct −640/+137/3′ Intron+1677/+3947). Replacement of the entire Flk-1 promoter including the 5′-UTR by the tk promoter in the transgenic construct also nearly abolished LacZ expression in the yolk sac (data not shown), and led to a reduced expression frequency in transgenic embryos (Table 1). Thus, the 5′-UTR might be involved in specifying Flk-1 expression in a subset of endothelial cells in the yolk sac.

The Flk-1 promoter is activated by HIF-2α. As shown above, the Flk-1 promoter (−640 bp/+299 bp) contains positive-acting regulatory sequences required for a strong and reproducible reporter gene transcription in transgenic mice. This suggests that transcription factors that are specifically expressed in endothelial cells activate the Flk-1 promoter in a cell-type–specific manner.

The basic helix-loop-helix PAS-domain transcription factor, HIF-2α (also known as HLF, HRF, or EPAR1), is prominently expressed in endothelial cells during mouse embryonic development19-21 and is thus a candidate regulator of Flk-1 expression. To determine if HIF-2α might be involved in the regulation of Flk-1 gene expression, we cotransfected A293 cells with a
luciferase reporter gene construct containing Flk-1 promoter sequences (−640 bp to +299 bp) and an eukaryotic expression vector that contained the mouse HIF-2α cDNA. In comparison to cells transfected with the luciferase reporter construct alone, cotransfection of the HIF-2α construct increased reporter gene activity approximately 15-fold (Fig 6). In contrast, HIF-1α, a close relative of HIF-2α that stimulates the hypoxia-induced transcription of the VEGF gene, failed to stimulate the reporter construct (Fig 6).

**DISCUSSION**

The murine Flk-1 receptor is crucial for the differentiation of the hemangioblastic lineage and during embryonic vascular development.1,2,6 Moreover, Flk-1 plays a central role in the regulation of neovascularization in a wide variety of tumors.34,35 To elucidate the basis of its endothelial expression, we have isolated and characterized regulatory sequences of the murine Flk-1 gene that confer endothelium-specific reporter gene expression in transgenic mouse embryos. Transgene expression driven by these sequences was strong, specific, and highly reproducible. Most importantly, we have shown that the isolated sequences were active in early-stage vascular development and may thus represent a clue towards the identification of the molecular mechanisms involved in hemangioblast differentiation and vasculogenesis. Moreover, transgene expression persisted until shortly after birth and was downregulated in adult animals, as it was described for the endogenous Flk-1 gene.13,28

The endothelium-specific expression in transgenic mouse embryos was mediated by a 939 bp fragment of the promoter region only in combination with a fragment of the first intron. 5′-flanking fragments up to −5.5 kbp alone were not sufficient to confer a reproducible endothelium-specific transgene expression. Reproducible endothelium-specific expression was therefore dependent on sequences from the first intron. These sequences also activated the heterologous tk promoter specifically in endothelial cells in vivo, and were active in an orientation-independent manner. Thus, they fulfill the criteria for an autonomous tissue-specific enhancer.
The endothelium-specific enhancer sequences were contained in a 510 bp intron fragment. Up to now, we have not observed endothelium-specific expression with shorter fragments, suggesting that multiple regulatory elements are clustered in this region. Several potential binding sites for transcription factors could be identified therein, including consensus binding sites for c-ets1, PEA3 (an Ets-like transcription factor), GATA transcription factors, and Scl/Tal-1. The c-ets1 transcription factor was proposed to be involved in the early differentiation of endothelial cells from their precursors, and c-ets1 is expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. Transcription factors of the GATA family are involved in the transcription of genes that are expressed in the hematopoietic and endothelial lineages, such as von Willebrand factor, and Scl/Tal-1 has recently been implicated in the regulation of Flk-1 expression in zebrafish. However, no direct effect of Scl/Tal-1 on Flk-1 expression has been observed so far in mice, although Scl-null mice have vascular defects.

Recently, analyses of the regulatory elements of other endothelium-specific genes such as von Willebrand factor, c-ets-1, or the endothelial receptors Tie1 and Tie2 have been reported. The most uniform vascular expression pattern reported was conferred by regulatory elements of the Tie2 gene. As it is the case for Flk-1, the first intron of the Tie2 gene also contains an autonomous endothelium-specific enhancer with potential binding sites of the Ets and GATA families. A major difference between the structural organization of the regulatory elements of the Flk-1 gene and the Tie2 gene is, however, that the Tie2 promoter by itself is active in certain embryonic blood vessels. Further studies will show whether common mechanisms are involved in the regulation of various endothelium-specific genes.

Analysis of Flk-1/LacZ knock-in mouse embryos that express the LacZ gene from the endogenous Flk-1 locus has previously shown that the LacZ reporter gene is expressed ubiquitously in the developing vasculature of E7.5 embryos. However, we have found that a fragment of the 5' UTR that is deleted in the knock-in construct is required for reporter gene expression in the yolk sac vasculature during later stages of embryonic development. In addition, deletion of this sequence between nucleotides +136 and +299 of the Flk-1 5' UTR in the transgenic construct reduced the expression frequency of the reporter gene. Based on transient transfection analyses in BAE cells, this sequence has been shown to contain a positive-acting, endothelial cell-specific element. Currently, we are investigating if proteins that specifically bind to the 5' UTR are involved in endothelial-specific transcription. The Flk-1 promoter appears to contain additional positive-acting regulatory sequences that are required for a strong and reproducible endothelium-
specific expression in the embryo proper. This assumption is supported by the observation that the heterologous tk promoter, when combined with the intron enhancer, showed significantly lower reporter gene expression levels and a reduced expression frequency in transgenic mouse embryos, when compared with the Flk-1 promoter. A positive activity of the Flk-1 promoter sequences has already been suggested by our previous studies in transfected BAE cells.\textsuperscript{17} Based on the stimulation of Flk-1 promoter activity in vitro and its endothelial expression, it seems likely that HIF-2α regulates the Flk-1 promoter in vivo. The involvement of HIF-2α in the regulation of Flk-1 expression further emphasizes the role of basic helix-loop-helix/PAS-domain transcription factors in the regulation of components of the VEGF signal transduction system and of vascular development. In particular, this has been shown in mouse embryos lacking functional genes for HIF-1α\textsuperscript{40} or ARNT\textsuperscript{41} that show defects in vascular development, perhaps due to reduced VEGF expression levels. HIF-2α is expressed most prominently in the endothelium of several developing organs, for example in the brain.\textsuperscript{20} It seems therefore likely that HIF-2α is involved in the regulation of Flk-1 expression in blood vessels that coexpress both HIF-2α and Flk-1. Interestingly, HIF-2α is also expressed in tissues that express the Flk-1 receptor ligand, VEGF, and has been shown to stimulate VEGF expression.\textsuperscript{19} Taken together, these observations support our hypothesis that HIF-2α is both an intrinsic and extrinsic regulator of blood vessel growth and function,\textsuperscript{20} by stimulating both receptor and ligand expression.

Among the endothelial receptor tyrosine kinases identified thus far, Flk-1 is the only receptor whose function is required for the determination of the endothelial lineage. Therefore, the Flk-1 gene represents the ideal candidate for studying the transcriptional regulatory mechanisms that are active during the emergence of the endothelial lineage. The observation that the isolated regulatory elements of the Flk-1 gene are active in early-stage vascular development is of great importance for this objective. Knowledge of the Flk-1 gene regulatory sequences is also of great potential relevance for the therapy of certain angiogenesis-dependent diseases, including cancer. Therefore, the study of the regulatory elements involved in the upregulation of Flk-1 expression in the tumor endothelium is particularly relevant for unraveling the mechanisms of tumor angiogenesis. The analysis of Flk-1 gene regulatory elements active in the tumor vasculature will provide a clue to the signaling pathways that could be targeted for antiangiogenic tumor therapy. Finally, the Flk-1 gene regulatory elements will be useful for targeting expression of genes to the vasculature, and the use of the Flk-1 gene regulatory elements in combination with the Cre/loxP system may provide a powerful tool for specifically inactivating genes in the developing vasculature or in tumor endothelium.

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Identification of Vascular Endothelial Growth Factor (VEGF) Receptor-2 (Flk-1) Promoter/Enhancer Sequences Sufficient for Angioblast and Endothelial Cell-Specific Transcription in Transgenic Mice

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