Journal of Cellular Biochemistry 54:56–66 (1994)

# Differential Expression of the Two VEGF Receptors flt and KDR in Placenta and Vascular Endothelial Cells

**Bernhard Barleon, Stefanie Hauser, Claudia Schöllmann, Karin Weindel, Dieter Marmé, Avner Yayon, and Herbert A. Weich**

Institute of Molecular Cell Biology, University of Freiburg, D-79108 Freiburg, Germany (B.B., S.H., C.S., K.W., D.M., H.A.W.); Department of Chemical Immunology, Weizmann Institute of Science, 76100 Rehovot, Israel (A.Y.)

**Abstract** Vascular endothelial growth factor (VEGF) is a newly identified growth and permeability factor with a unique specificity for endothelial cells. Recently the flt-encoded tyrosine kinase was characterized as a receptor for VEGF. A novel tyrosine kinase receptor encoded by the KDR gene was also found to bind VEGF with high affinity when expressed in CMT-3 cells. Screening for flt and KDR expression in a variety of species and tissue-derived endothelial cells demonstrates that flt is predominantly expressed in human placenta and human vascular endothelial cells. Placenta growth factor (PlGF), a growth factor significantly related to VEGF, is coexpressed with flt in placenta and human vascular endothelial cells. KDR is more widely distributed and expressed in all vessel-derived endothelial cells. These data demonstrate that cultured human endothelial cells isolated from different tissues express both VEGF receptors in relative high levels and, additionally, that all investigated nonhuman endothelial cells in culture are also positive for KDR gene expression. © 1994 Wiley-Liss, Inc.

**Key words:** VEGF, PlGF, KDR, flt, endothelial cells, placenta

Vascular endothelial growth factor (VEGF) [1–4], also known as vascular permeability factor (VPF) [5,6] or vasculotropin (VAS) [7], is a member of a recently identified family of endothelial cell mitogens and angiogenic factors. VEGF was purified from media conditioned by several tumor cell lines [3,7–9] and also by bovine pituitary folliculostellate cells [1,4]. Molecular cloning of the cDNA suggests that in human cells at least four species of VEGF can occur as 121, 165, 189, and 206 amino acid forms, generated by alternative splicing of the VEGF gene [2,10].

Another member of the VEGF family is the recently identified placenta growth factor (PlGF), which was, so far, found only in placenta and choriocarcinoma cells [11]. PlGF, like VEGF, is a secretory, dimeric, and N-glycosylated protein. PlGF and VEGF show high amino acid sequence similarity in the PDGF-like region. Thus, the PlGF protein shows a similarity of approximately 70% to the PDGF-like domain of human VEGF when both the identical and conservative amino acid changes are considered. Like VEGF, PlGF also stimulates the growth of endothelial cells [11].

VEGF is a growth factor with a unique specificity for vascular endothelial cells. The mitogenic activity of VEGF appears to be mediated by specific VEGF receptors which are found on the surface of various types of endothelial cells [12–15] or can be induced in vivo in highly malignant brain tumors [16]. High-affinity binding sites for VEGF were found on the cell surface of bovine endothelial cells [12] and human umbilical vein endothelial (HUVE) cells [17], as well as on various fetal and adult endothelial cell strains [15]. Colocalization of $^{125}$I-VEGF binding with Factor VIII–like immunoreactivity demonstrated binding sites associated with vascular endothelial cells of both fenestrated and nonfenestrated microvessels and the endothelium of large vessels. No displaceable binding was evident on nonendothelial cells [18].

Received March 17, 1993; accepted August 24, 1993.

Address reprint requests to Herbert A. Weich, Department of Gene Expression, Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany.

Herbert A. Weich's present address is Department of Gene Expression, Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany.

© 1994 Wiley-Liss, Inc.

Recently a receptor-type tyrosine kinase encoded by the flt gene [19] was shown to bind VEGF with high affinity [20]. The overall structure of the deduced amino acid sequence indicated that flt is closely related to the fms family, to which belong colony stimulating factor-1 (CSF-1) receptor gene [21,22], platelet-derived growth factor (PDGF) receptor genes [23,24], and the c-kit gene [25–27]. The gene was therefore designated as flt (*f* ms-*l*ike *t*yrosine kinase) gene. The flt gene is also highly homologous to the very recently cloned human Flt4 gene [28]. The mRNA of flt is expressed in a variety of normal tissues of human and rat with high expression in human placenta, but not in most of the tumor cell lines so far examined [19].

A partial cDNA encoding a novel receptor tyrosine kinase was recently cloned from a human endothelial cell cDNA library by polymerase chain reaction (PCR) using a primer for conserved receptor tyrosine kinase regions. The predicted amino acid sequence contained multiple characteristics typical of a type III receptor tyrosine kinase and was therefore designated KDR for *k*inase insert *d*omain–containing *r*eceptor. Expression of the receptor was detected in bovine endothelial cells but not in smooth muscle cells or fibroblasts [29]. Very recent results indicate that KDR encodes a second receptor for VEGF [30]. The homologous gene for the human KDR gene in mouse is Flk1 [31] and for rat is TKrC [32], whereas the mouse and rat homologous genes for flt are not known.

We have investigated the expression pattern of the KDR and flt gene as well as that of the PlGF gene by Northern blot analysis in a large number of different endothelial cell types and human and fetal bovine placental tissue. We found that KDR is widely expressed in different vascular endothelial cell types but the expression rate is low in human and fetal bovine placental tissue. In contrast, high flt expression was detectable in human endothelial cells and human placental tissue. High expression of PlGF was found in human placental tissue and in human vascular endothelial cells.

## MATERIALS AND METHODS

### Cell Lines

Primary human umbilical vein endothelial cells (HUVE) were isolated and cultured as described [33]. Additionally, HUVE cells were also purchased from PromoCell (Heidelberg, Germany) and cultured according to the provided protocol. Human aortic endothelial cells (HAEC) and human microvessel endothelial cells (MVEC) were grown in M199 supplemented with 25 mM HEPES, 10% human serum, 10% newborn calf serum (heat inactivated), 90 μg/ml heparine, and 20 μg/ml endothelial cell mitogen using fibronectin- or gelatin-coated cultured flasks. Human smooth muscle cells were isolated and cultured as described [34]. Bovine aortic endothelial cells (BAE), bovine adrenal capillary endothelial cells (BACE), and bovine brain capillary endothelial cells (BBC) [14] were grown in DMEM containing 10% fetal calf serum (FCS) endothelial mitogen using gelatinized culture flasks. Bovine fetal aortic endothelial cells (FBAE) were grown in DMEM containing 10% FCS. Porcine aortic endothelial cell line (PAE) were cultured in Ham's F12 medium containing 10% FCS [35]. All other cell lines were grown in DMEM supplemented with 10% calf serum.

### Generation of cDNA Probes

The following sense and antisense primers were used for gene amplification. (1) KDR gene: sense primer KD1: 5′-CAACCTTCTAGGTGCCTGTAC, antisense primer KD2: 5′-GGATATTTCGTGCCGCCTGG. PCR product corresponds to nucleotides 1,188–1,594 of the published human cDNA KDR sequence [30]. (2) flt gene: sense primer VR1: 5′-CCCAAGCTTAGAGGACGGACTCTGGC, containing a *Hind* III site, antisense primer VR2: 5′-GAAGATCATTGCACTGAGGTGTTAAC, containing a *Bgl* II site. PCR product corresponds to nucleotides 172–1,231 of the human flt cDNA sequence [19]. (3) PlGF gene: sense primer 310: 5′-ACGTCTGAGAAGATGCCGGT, antisense primer 821: 5′-AGGGAAACAGTTGGCTAATAA. PCR product corresponds to nucleotides 309–884 of the human PlGF cDNA sequence [11].

### Preparation of Specific cDNA Probes

Total RNA was isolated as described [36]. RNA (1 μg) from BACE for KDR gene and from human placenta for PlGF gene were used for cDNA amplification with PCR as described [37], with the exception that the reactions finally were incubated for 10 min at 72°C. The PCR products were separated on an agarose gel and subsequently cloned in the *Sma* I site of the Bluescript vector (Stratagene, La Jolla, CA). For the flt gene, 2 μl of a human placenta λgt11

cDNA library ($1–9 \times 10^9$ pfu/ml, Clontech Laboratories, Palo Alto, CA) was subjected to PCR. The reaction mixture (100 μl) was 67 mM Tris HCl, pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 250 μM dNTP, 0.17 mg/ml BSA, 0.5 mM of each primer, and 2 U Taq polymerase (Promega). After 30 cycles of 1.5 min at 94°C, 1.5 min at 65°C, and 4 min at 72°C, the reaction product was extracted with phenol and phenol chloroform (1:1) and ethanol precipitated. The PCR products were dissolved and filled in using Klenow fragment and subcloned into *Eco* RV site of the Bluescript vector. Positive clones were selected by small-scale preparations of plasmid DNA as described elsewhere [38], followed by restriction analysis. The DNA of recombinant clones were amplified by large-scale plasmid preparation, analyzed, and identified by automatic sequencing from both sites [39].

### Northern Blot Analysis

Total RNA was prepared from various cell lines and tissues by a guanidin/cesium chloride centrifugation method [36] or by a modification of the acid guanidinium thiocyanate-phenolchloroform extraction protocol [40]. Northern blot analysis was done as described [37]. For flt gene Northern blot was also carried out with low stringency as described [28]. Final wash was with $1 \times$ SSC at 50°C.

### VEGF Receptor Binding Assay

Binding experiments were performed with recombinant human $VEGF_{165}$ on BACE cells by a modification of the procedure as described previously for smooth muscle cells [34]. Briefly, cells were seeded in DMEM–10% calf serum at $5 \times 10^4$ cells per well in 12-well plates. After 72 h, the cells were washed with binding buffer (DMEM, 25 mM HEPES, 1 mg/ml bovine serum albumin, pH 7.4), and then incubated for 3 h at 4°C with binding buffer containing increasing concentrations of $^{125}$I-rh-$VEGF_{165}$ [41]. Unspecific binding for each concentration was determined by addition of a 100-fold excess of unlabeled ligand. Cultures were washed extensively with binding buffer and then solubilized with 0.3 M NaOH, 0.1% SDS. Radioactivity present in the lysates was quantitated using a gamma counter. Recombinant human $VEGF_{165}$ was iodinated to 300,000–400,000 cpm/ng by using the chloramin T method [42]. The data were subjected to Scatchard plot analysis [43,44].

### Assay for Mitogenic Activity

Mitogenic assays were performed as described previously [45]. Briefly, BAE cells were seeded at $1 \times 10^4$ cells per well into 24-well plates in DMEM–10% calf serum. Cells were allowed to attach overnight at 37°C. The medium was changed to DMEM–2% calf serum supplemented with 1.5 μM thymidine. Mitogens were added 24 h later. Incubation was continued for an additional 18 h, after which time 1 μCi [$^3$H] thymidine (56.7 Ci/mmol, NEN) was added. Cells were kept at 37°C for an additional 6 h. Cell monolayers were fixed with methanol, washed with 5% trichloroacetic acid, solubilized in 0.3 M NaOH, and counted by liquid scintillation.

## RESULTS

### Expression Pattern of KDR Gene

Because very little is known about KDR expression and distribution in cells and tissues, we investigated the expression pattern of KDR in cultured vascular endothelial cells of different origin, some nonendothelial cell lines as well as in human and fetal bovine placenta. The polymerase chain reaction was used to amplify the specific cDNA probe for the KDR gene containing the full kinase insert domain (nucleotide 1,188–1,594) [30]. The expression pattern of the KDR gene was examined by Northern blot analysis using stringent conditions for hybridization. The expression of the KDR gene was found to be specific for endothelial cells from many different sources, but was not found in other cell types (Fig. 1). KDR is also expressed in human and fetal bovine placenta at a low rate. The KDR mRNA is visible as a main band of approximately 7.0 kb (Fig. 1). The size of the mRNA species is in agreement with earlier reports [29]. Additional bands were visible after a longer exposure. The same results were obtained using a human 1.4 kb cDNA fragment, corresponding to nucleotides 360–1,720 of the full-length sequence [30] coding for the extracellular part of the KDR receptor (not shown). However, compared to the flt gene (see Fig. 2), the expression level of KDR was generally lower and a 10-fold longer exposure time was necessary to get a similar signal on the autoradiogram.

### Expression Pattern of flt Gene

It was known that flt gene is expressed in a variety of normal tissues of human and rat [19], but little is known about the expression pattern

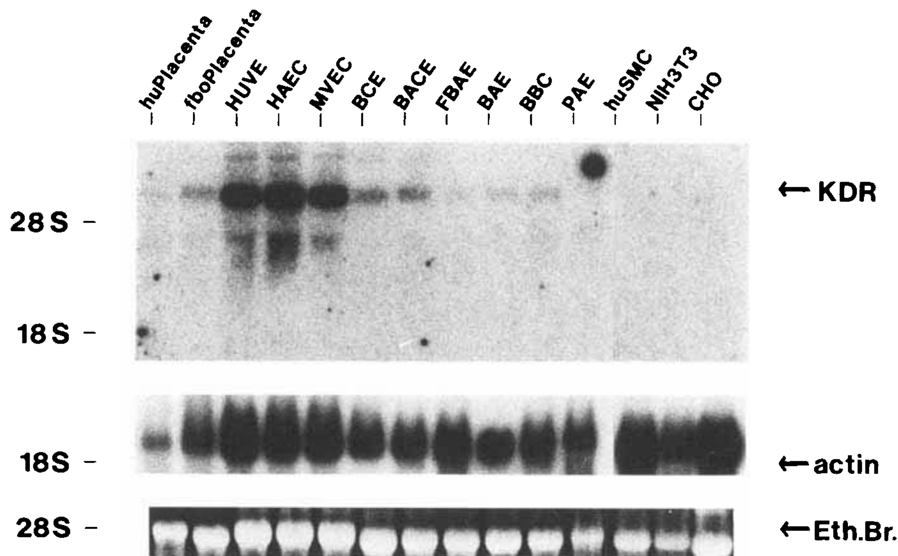


**Fig. 1.** Expression of KDR gene in various vascular endothelial cell types from different sources, some nonendothelial cell lines and human and fetal bovine placenta. About 8 μg of total RNA for each lane were separated on an agarose gel, transferred to nitrocellulose filter, and hybridized with a specific cDNA probe (see Materials and Methods). For KDR gene, hybridization was done with a 0.4 kb cDNA fragment **(top panel). Lane 1:** huPlacenta, human placenta; **lane 2:** fboPlacenta, fetal bovine placenta; **lane 3:** HUVE, human umbilical vein endothelial cells; **lane 4:** HAEC, human aortic endothelial cells; **lane 5:** MVEC, human microvessel endothelial cells; **lane 6:** BCE, bovine capillary endothelial cells; **lane 7:** BACE, bovine adrenal capillary endothelial cells; **lane 8:** FBAE, fetal bovine aortic endothelial cells; **lane 9:** BAE, bovine aortic endothelial cells; **lane 10:** BBC, bovine brain capillary endothelial cells; **lane 11:** PAE, porcine aortic endothelial cell line; **lane 12:** boSMC, bovine smooth muscle cells; **lane 13:** NIH3T3, mouse 3T3 fibroblasts; **lane 14:** CHO, Chinese hamster ovarium cells. To control for sample loading and RNA integrity, all filters were stripped and rehybridized with a full-length mouse α-actin cDNA probe **(lower panel).** The ethidium bromide stain of the 28-S RNA of the gels is also shown. Exposure time was 21 days (with amplifier).

in different endothelial cell types. We therefore investigated the expression pattern of the flt gene in vascular endothelial cells of different sources, some nonendothelial cell lines and human and fetal bovine placenta. The polymerase chain reaction was used to amplify a part of the extracellular domain (nucleotide 172–1,231) of the published cDNA sequence [19]. Using Northern blot analysis, the flt gene was shown to be highly expressed in human placental tissue and, to a significant lower extent, in all human endothelial cells used in this investigation (Fig. 2). In placenta, three bands with approximately 7.5, 3.4, and 2.7 kb were visible, the 3.4 kb mRNA species being the dominant band. The size of the transcripts are consistent with earlier published results, which shows that the 7.5 kb band corresponds to the full-length receptor mRNA and that the lower bands are probably corresponding to mRNAs for truncated forms containing the extracellular part of the receptor [19]. In the human endothelial cells the pattern is different. There are four mRNA species: the approximately 8.0 kb and 7.5 kb bands appearing as a doublet and two bands at 3.4 kb and 2.7 kb. The predominant species are the 8.0 kb and 7.5 kb bands (Fig. 2). There is also a very faint signal in the porcine aortic endothelial cell line (PAE), after an exposure of one week. All bovine vascular endothelial cells as well as bovine maternal and fetal placental tissues were negative for detection of flt gene expression, whereas mouse placenta and some rat tissues (e.g., lung, kidney) were positive for flt gene expression after prolonged exposure (not shown). We were not able to get a positive signal for flt gene expression in bovine-derived RNAs even under low stringency conditions and after more than one week exposure time of the autoradiogram (not shown).

### Binding Studies With $^{125}$I-VEGF$_{165}$ and Thymidine Incorporation Assay

In earlier reports it was shown that bovine brain capillary endothelial (BBC) cells [14] and aortic arch-derived bovine endothelial (ABAE) cells [12] have two high affinity binding sites for VEGF. To confirm that the bovine endothelial cells used in our studies for gene expression have also two high affinity binding sites for

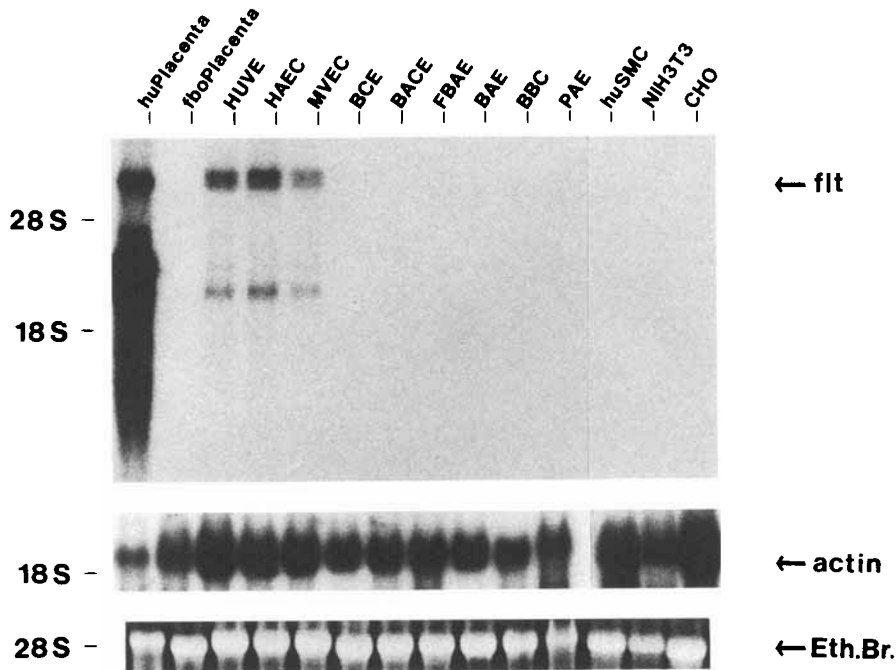


**Fig. 2.** Expression of flt gene in various types of vascular endothelial cells from different sources, some nonendothelial cell lines and human and fetal bovine placenta. About 8 μg of total RNA for each lane were separated on an agarose gel, transferred to nitrocellulose filter, and hybridized with a specific cDNA probe (see Materials and Methods). For flt gene, hybridization was carried out with a 1.05 kb cDNA fragment. To control for sample loading and RNA integrity, all filters were stripped and rehybridized with a full-length mouse α-actin cDNA probe (below). The ethidium bromide stain of the 28-S RNA of the gel is also shown. Exposure time was 2.5 days (with amplifier).

VEGF, we performed binding studies with radiolabeled, purified recombinant human VEGF (rh-$VEGF_{165}$) [41] on bovine adrenal capillary endothelial (BACE) cells. Half maximal and maximal binding occurred at 12 pM and 125 pM, respectively (Fig. 3). It was possible to displace the bound growth factor almost totally by adding an excess (100 times) of unlabeled $VEGF_{165}$. The saturation of the specific binding in the pM-range indicates, that $VEGF_{165}$ is able to bind to the high affinity receptors described for VEGF on bovine endothelial cells [12–14]. Scatchard analysis of the binding data demonstrates the existence of two different receptor classes: the first type has a $K_D$ of 6.4 pM (2,160 receptors/cell) and the second type has a $K_D$ of 20 pM (3,100 receptors/cell). We obtained similar results with fetal bovine aortic endothelial (FBAE) cells and with bovine aortic endothelial (BAE) cells (not shown).

These data suggest that the bovine endothelial cells used in this study have two VEGF receptors but that the human cDNA probe either does not cross-hybridize with the bovine flt mRNA or that bovine endothelial cells express a different VEGF binding receptor which is not yet identified. It was shown before that recombinant $VEGF_{165}$ from insect cells is indistinguishable in receptor binding if compared with mammalian VEGF [46]. Thymidine incorporation experiments as well as proliferation studies were carried out on BAE cells to investigate whether our purified $VEGF_{165}$ is biologically active. $VEGF_{165}$ at concentrations between 3 and 5 ng/ml (0.075–0.125 nM) had a maximal effect on DNA synthesis (Fig. 4). Similar results were obtained with other bovine vascular endothelial cells, for example, with FBAE cells and BACE cells (data not shown).

## Sequence Homology Studies

Because we were not able to get a positive flt signal in bovine cells and tissues with our human probe, we compared the homology between the different VEGF receptors to find out the degree of conservation in different species. We found that the homology between the homologous VEGF receptor genes KDR (human) [30], Flk1 (mouse) [31], and TKrC (rat) [32] was in the range of 90–98% within the catalytic domain. The homology within the extracellular part between KDR and Flk1 was about 80%

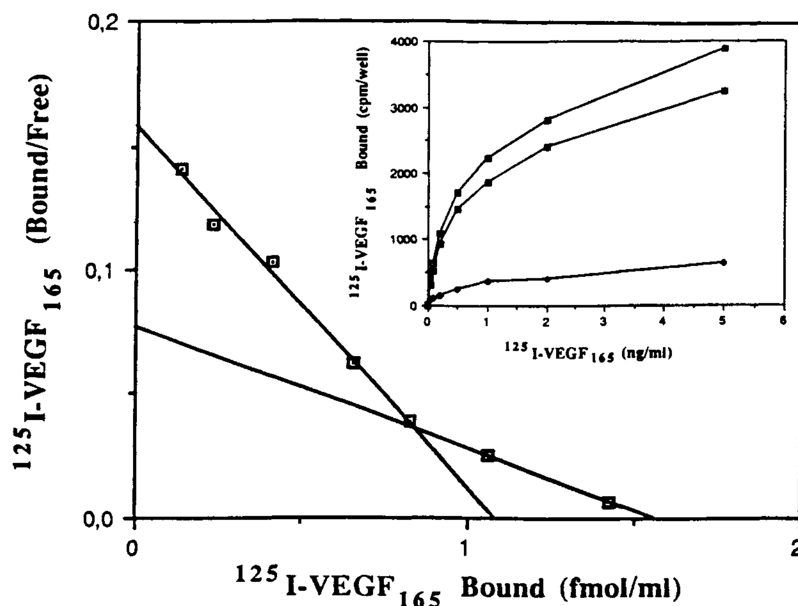


**Fig. 3.** Saturation binding curve and Scatchard plot of $^{125}$I-VEGF$_{165}$ binding to BACE cells. Binding was carried out on confluent BACE cells in 12-well plates for 3 hr at 4°C in the presence of increasing concentrations of $^{125}$I-VEGF$_{165}$ (inset). The nonspecific binding (closed dots) was determined in parallel dishes containing 100-fold excess of unlabeled VEGF$_{165}$ and was substracted from the total binding (open squares) to obtain the specific binding (closed squares). Binding results were analyzed by the method of Scatchard [46] with the aid of the LIGAND program [47] to fit the data to a two-site model.

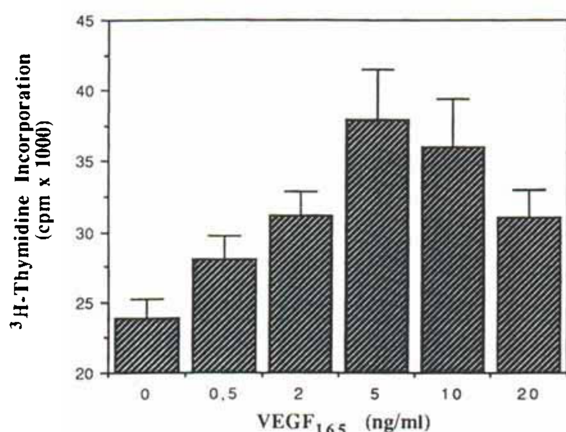


**Fig. 4.** Stimulation of [$^3$H]-thymidine uptake by bovine aortic endothelial (BAE) cells after incubation with different amounts of purified recombinant human VEGF$_{165}$. Cells were starved (2% bovine calf serum) for at least 24 hr before treatment. Then growth factor was added and cells were incubated for 18 hr before addition of [$^3$H]-thymidine. Cells were harvested and counted 6 hr later.

(Table I). This result indicated that at least this type of VEGF receptor is highly conserved between different species. The homology between KDR and the two other known human VEGF receptor genes flt [19] and Flt4 [47] was 80% and 77% for the amino half and 79% for the carboxyl half of the kinase domain, respectively. The homology to the kinase insert was 45% and 12%, the homology within the extracellular domain was 33% and 37%, respectively.

Sequence homology with other kinases was significantly lower, ranging between 56–59% for the amino half and 51–57% homology for the carboxyl half of the receptor tyrosine kinase. The homology to the kinase insert was in the range of only 7% to 14% (Table I).

### Expression Pattern of PlGF Gene

PlGF is a protein closely related to VEGF and so far no data were reported about expression and distribution of this growth factor in endothelial cells and tissues. We used RNA from the same cells to investigate the expression pattern of this growth factor. The specific cDNA probe for PlGF corresponds to nucleotides 309–884 of the human PlGF cDNA sequence [11]. As expected for a cDNA isolated from a placenta library, PlGF was expressed as a 1.8 kb band in human placenta tissue and, surprisingly, in all human vascular endothelial cells (Fig. 5). There was also a faint signal in most of the bovine vascular endothelial cells after a longer exposure (not shown). Interestingly, the flt and PlGF gene expression level was very high and colocalized in placental tissue and the human vascular

**TABLE I. Amino Acid Homology in Tyrosine Kinase Domain Between KDR Gene and Other Type III Tyrosine Kinase Receptor Genes**

| Genes | Amino half[a] % | Insert[b] % | Carboxyl half[c] % | Extracellular domain[d] % |
|---|---|---|---|---|
| VEGF receptor genes | | | | |
| KDR/FLK-1 | 95 | 90 | 97 | 80 |
| KDR/TKrC | 96[e] | 91 | 98[e] | |
| KDR/FLT4 | 77 | 12 | 79 | 33 |
| KDR/flt | 80 | 45 | 79 | 37 |
| Unrelated receptor pairs | | | | |
| KDR/Flt3 (FLK2) | 58 | 17 | 55 | |
| KDR/PDGF-αR | 58 | 14 | 57 | |
| KDR/PDGF-βR | 56 | 10 | 51 | |
| KDR/CSF-1 | 58 | 11 | 52 | |
| KDR/c-kit | 59 | 7 | 54 | |
| Related receptor pairs | | | | |
| PDGF-αR/PDGF-βR | 84 | 30 | 71 | |
| Bek/flg | 87 | 50 | 92 | |

[a]KDR residue 820–929; [b]930–1000; [c]1001–1229; [d]KDR residue 1–765.
[e]Only partly published
KDR [30]; FLK-1 [28]; TKrC [32]; FLT4 [28]; flt [19]; Flt3 [54]; FLK2 [55]; PDGF-αR [24]; PDGF-βR [23]; c-kit [26]; CSF-1 [21]; Bek/flg [56].

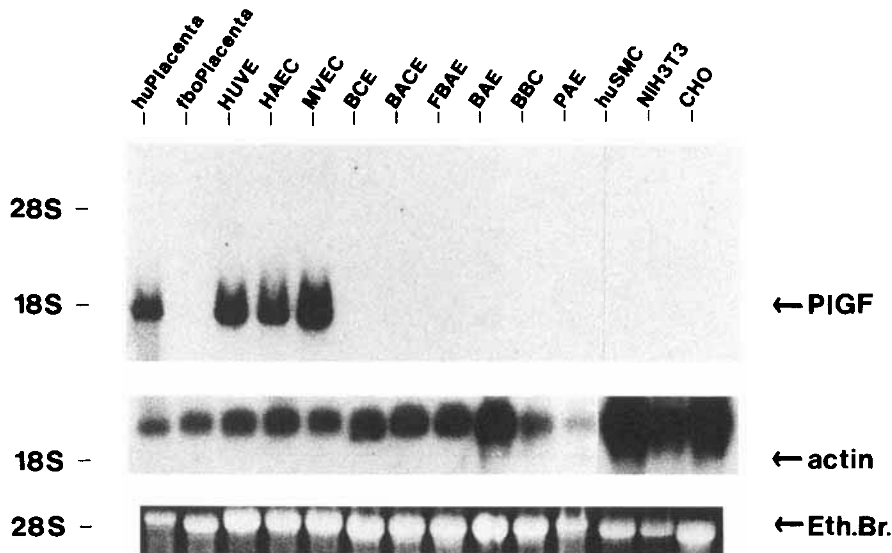


**Fig. 5.** Expression of PlGF gene in various vascular endothelial cell types from different sources, some nonendothelial cell lines and human and fetal bovine placenta. About 8 μg of total RNA for each lane was separated on an agarose gel, transferred to nitrocellulose filter, and hybridized with a specific cDNA probe (see Materials and Methods). For PlGF gene, hybridization was carried out with a 0.56 kb cDNA fragment. To control for sample loading and RNA integrity, all filters were stripped and rehybridized with a full-length mouse α-actin cDNA probe. The ethidium bromide stain of the 28-S RNA of the gels is also shown. Exposure time was nine days (with amplifier).

endothelial cells. No expression was detected in fibroblasts or in vascular smooth muscle cells.

## DISCUSSION

The identification and characterization of receptors which mediate the signals of mitogenic and angiogenic growth factors, such as VEGF and PlGF, is of general interest because of the potential involvement of those factors in physiological and pathological processes such as blood vessel formation and tumor angiogenesis. For VEGF, the presence of high affinity binding sites was demonstrated on endothelial cells of many different sources. In situ ligand binding

studies with whole body sections also indicated that VEGF binding correlates with the detection of von Willebrand factor, an endothelial specific antigen marker [18]. Very recently, the receptor-type tyrosine kinases flt and KDR were identified as receptors for VEGF [20,30]. The flt gene, isolated from a placenta library, was reported to be expressed in several normal tissues of adult human and rat, with no clear identification of the expressing cell type [19]. KDR was isolated from a human endothelial cell cDNA library and known to be expressed in bovine endothelial cells [29].

Our Northern blot studies with several endothelial cells and nonendothelial cell lines as well as human and fetal bovine placenta revealed that the flt gene is highly expressed in human placental tissue and in all other human vascular endothelial cells investigated. Unexpectedly, all of our cultured bovine vascular endothelial cell types including placental tissue were negative for flt gene expression. The reason for this negative result is not known. First, there may be no cross-hybridization between the human cDNA and the bovine mRNA for the receptor gene. On the other hand, the human flt cDNA fragment gives a positive signal with porcine aortic endothelial cells, mouse placenta, and with several rat tissues if exposed over a longer period. This indicates that the flt gene seems to be conserved in several species. This assumption is further supported by the finding that the Flt4 gene, another putative receptor for VEGF [47], is also highly conserved in different species including chicken [28]. Another support is the isolation of two partial tyrosine kinase receptor cDNA clones (CK1 and I31) from avian embryo. These clones show a high amino acid sequence homology (85–96%) to the human VEGF receptor genes [48], especially for KDR and Flt4. Since the expression pattern of both genes as well as that of the flt gene is similar, with highest expression in lung tissue, it is not possible to determine which, if either, of CK1 and I31 are the corresponding avian homologous genes for two of the known human VEGF receptor genes. The second possibility, which seems to be very unlikely, is that the flt gene is not expressed in cultured bovine vascular endothelial cells and in bovine placental tissue. Results from Sarzani and coworkers [32] seem to confirm the second possibility. They showed that flt is not expressed in cultured bovine aortic endothelial cells. However, low expression of the flt gene was detectable with a human flt cDNA probe, if fresh bovine aortic intima was used for RNA preparation [32].

The cDNA probe for the KDR receptor, which was generated with RNA from bovine aortic endothelial cells (BACE), hybridized with human vascular endothelial cells as well as with the RNA from endothelial cells of other species (e.g., porcine, rat). These results were confirmed with a human cDNA fragment for the KDR receptor. Northern blot analysis revealed that the KDR gene is expressed in nearly all vascular endothelial cells examined, even though the expression level is very different between cells, and generally lower. KDR is also expressed in human and fetal bovine placental tissue but not in nonendothelial cells. These cell types were known to be unresponsive for VEGF stimulation [7,15], which is consistent with the former finding that VEGF is an endothelial cell–specific mitogen [1,2,6,7,49]. In summary, our data about the differential expression of the two VEGF receptor mRNAs are based on Northern blot analysis. Further studies are necessary to verify the differential presence of these receptor proteins in vascular endothelial cells.

Our binding studies and previously published results [12–15,17] showed two high-affinity binding sites for VEGF in vascular endothelial cells. The second VEGF receptor, KDR, seems to be more specific for endothelial cells, because for flt it was shown before that this gene is also expressed in some nonendothelial cell lines, for example, human choriocarcinoma cells [19]. This assumption is further supported by our preliminary studies with monocytes, which express the flt receptor but not the KDR receptor [B. Barleon, unpublished observations]. Even if our results may favor the idea that flt and KDR are the receptors for VEGF binding in endothelial cells, we cannot exclude the possibility that Flt4, the recently cloned putative third VEGF receptor, or other as yet unidentified receptors for VEGF and PlGF may exist in these cells.

More interesting is the high expression of the PlGF gene in human endothelial cells. PlGF has similar characteristics with VEGF, for example, the amino acid sequence is nearly identical in size with $VEGF_{121}$ and there is a high similarity between PlGF and VEGF in the PDGF-like domain of the VEGF family [11,50]. This region is probably responsible for the activities of the different VEGF isoforms [6] and therefore PlGF might have activities very similar to VEGF [11]. Because it is already known that PlGF is a

mitogen for endothelial cells [11,50], an autocrine loop for PlGF may exist in these cells. There are several reports about autocrine stimulation of growth factors or growth stimulation via a positive autocrine signal in vascular endothelial cells, which may serve as an amplifier of the mitogenic response under certain physiological conditions, for example, wound healing and new capillary formation [51,52]. Experiments are under way to prove the existence of the postulated autocrine loop for PlGF in vascular endothelial cells by immunological detection of secreted PlGF from HUVE cell–conditioned media. In this respect, based on the high similarity between PlGF and VEGF, the high coexpression of flt and PlGF in placenta and human vascular endothelial cells indicates that this autocrine loop may be mediated via the flt/VEGF receptor and not via the second VEGF receptor KDR. However, binding experiments with purified PlGF have to be performed to analyze if the PlGF protein binds to the flt receptor with high affinity. Finally, vascular endothelial cells are remarkably heterogenic concerning differences between surface antigens as well as their responsiveness to different growth factors [53]. Our results demonstrate a marked difference in gene expression rates which may contribute to both structural and functional heterogeneity of vascular endothelial cells.

**Note added in proof.** While this manuscript was in preparation, the murine genes for the flt and FLT-4 receptors were cloned [57]. For flt the overall sequence identity is 82%, the sequence identity of the extraplasmatic region is about 79%. This result shows that the flt gene is highly conserved between the mouse and human genome.

## ACKNOWLEDGMENTS

We thank Bruce Terman for advice and the human KDR probe, and Roy Bicknell for carefully reading the manuscript. Additionally, we thank Drs. Victor van Hinsbergh, Carl-Henrik Heldin, Jean Plouët, and Roy Bicknell for the generous gift of endothelial cells. This work was supported by a grant from the German Secretary of Research and Technology, No. 01 GA 8816.

## REFERENCES

1. Ferrara N, Henzel WJ (1989): Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858.
2. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989): Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309.
3. Conn G, Bayne ML, Soderman DD, Kwok PW, Sullivan KA, Palisi TM, Hope DA, Thomas KA (1990): Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to the platelet-derived growth factor. Proc Natl Acad Sci USA 87:2628–2632.
4. Tischer E, Gospodarowicz D, Mitchell R, Silva M, Schilling J, Lau K, Crisp T, Fiddes JC, Abraham JA (1989): Vascular endothelial growth factor: A new member of the platelet-derived growth factor gene family. Biochem Biophys Res Commun 165:1198–1206.
5. Senger DR, Perruzzi CA, Feder J, Dvorak HF (1986): A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 46:5629–5632.
6. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989): Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246: 1309–1312.
7. Plouët J, Schilling J, Gospodarowicz D (1989): Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. EMBO J 8:3801–3806.
8. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J (1989): Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 84:1470–1478.
9. Connolly DT, Olander JV, Heuvelman D, Nelson R, Monsell R, Siegel N, Haymore BL, Leimgruber R, Feder J (1989): Human vascular permeability factor: Isolation from U937 cells. J Biol Chem 264:20017–20024.
10. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW (1991): The vascular endothelial growth factor family of polypeptides. J Cell Biochem 47:211–218.
11. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG (1991): Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA 88:9267–9271.
12. Vaisman N, Gospodarowicz D, Neufeld G (1990): Characterization of the receptor for vascular endothelial growth factor. J Biol Chem 265:19461–19466.
13. Plouët J, Moukadiri H (1990a): Characterization of the receptor to vasculotropin on bovine adrenal cortex–derived capillary endothelial cells. J Biol Chem 265: 22071–22074.
14. Plouët J, Moukadiri H (1990b): Specific binding of vasculotropin to bovine brain capillary endothelial cells. Biochimie 72:51–55.
15. Olander JV, Connolly DT, DeLarco JE (1991): Specific binding of vascular permeability factor to endothelial cells. Biochem Biophys Res Commun 175:68–76.
16. Plate K, Breier G, Weich HA, Risau W (1992): Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845–848, 1992.
17. Bikfalvi A, Sauzeau C, Moukadiri H, Maclouf J, Busso N, Bryckaert M, Plouët J, Tobelem G (1991): Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: Bind-

ing, internalization, degradation, and biological effects. J Cell Physiol 149:50–59.
18. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N (1992): Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89:244–253, 1992.
19. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M (1990): Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5:519–524.
20. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT (1992): The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989–991, 1992.
21. Hampe A, Gobet M, Sherr CJ, Galibert F (1984): Nucleotide sequence of the feline retroviral oncogene v-fms shows unexpected homology with oncogenes encoding tyrosine-specific protein kinases. Proc Natl Acad Sci USA 81:85–89.
22. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanly ER (1985): The c-fms proto-oncogene product is related to the receptor for mononuclear phagocyte growth factor, CSF-1. Cell 41:665–676.
23. Yarden Y, Escobedo JA, Kuang WJ, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Fried VA, Ullrich A, Williams LT (1986): Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323:226–232.
24. Matsui T, Heideran M, Miki T, Popescu N, La Rochelle W, Kraus M, Pierce J, Aaronson S (1989): Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science 243:800–805.
25. Besmer P, Murphy JE, George PC, Qui F, Bergold PJ, Lederman L, Snyder HW, Brodeur D, Zuckerman EE, Hardy WD (1986): A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature 320:415–421.
26. Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A (1987): Human proto-oncogene c-kit: A new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6:3341–3351.
27. Qui F, Ray P, Brown K, Barker PE, Jhanwar S, Ruddle FH, Besmer P (1988): Primary structure of c-kit: Relationship with the CSF-1/PDGF receptor kinase family–oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. EMBO J 7:1003–1011.
28. Pajusola K, Aprelikova O, Korhonen J, Kaipainen A, Perlovaara L, Alitalo R, Alitalo K (1992): Flt4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. Cancer Res 52:5738–5743.
29. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB (1991): Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 6:1677–1683.
30. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Böhlen P (1992): Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. Biochem Biophys Res Commun 187:1579–1586.
31. Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR (1991): A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genic linkage to *c-kit*. Proc Natl Acad Sci USA 88:9026–9030.
32. Sarzani R, Arnaldi G, De Pirro R, Moretti P, Schiaffino S, Rappelli A (1992): A novel endothelial tyrosine kinase cDNA homologous to plateled-derived growth factor receptor cDNA. Biochem Biophys Res Commun 186: 706–714.
33. Weich HA, Iberg N, Klagsbrun M, Folkman J (1990): Expression of acidic and basic fibroblast growth factors in human and bovine vascular smooth muscle cells. Growth Factors 2:313–320.
34. Schöllmann C, Grugel R, Tatje D, Hoppe J, Marmé D, Weich HA (1992): Basic FGF modulates the mitogenicity of the PDGF isoforms by specific upregulation of the α-type receptor in vascular smooth muscle cells. J Biol Chem 267:18023–18039.
35. Westermark B, Siegbahn A, Heldin CH, Claesson-Welsh L (1990): B-type receptor for platelet-derived growth factor mediates a chemotactic response by means of ligand-induced activation of the receptor protein-tyrosine kinase. Proc Natl Acad Sci USA 87:128–132.
36. Chirgwin JM, Pryzyba AE, MacDonald RJ, Ruttner WJ (1979): Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299.
37. Weindel K, Marmé D, Weich HA (1992): Aids-associated kaposi's sarcoma cells in culture express vascular endothelial growth factor. Biochem Biophys Res Commun 183:1167–1174.
38. Birnboim HC, Doly J (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1519.
39. Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.
40. Xie WQ, Rothblum LI (1991): Rapid, small-scale RNA isolation from tissue culture cells. BioTech 11:325–327.
41. Fiebich B, Jäger B, Schöllmann C, Weindel K, Wilting J, Kochs G, Marmé D, Hug H, Weich HA (1992): Synthesis and assembly of a functionally active human vascular endothelial growth factor homodimers in insect cells. Eur J Biochem 211:19–26.
42. Hunter W, Greenwood FC (1962): Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194:495–496.
43. Scatchard G (1949): The attraction of proteins for small molecules and ions. Ann NY Acad Sci 51:660–672.
44. Munson PJ, Rodbard D (1980): LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239.
45. Miyazono K, Okabe T, Urabe A, Takaku, Heldin CH (1987): Purification and properties of an endothelial cell growth factor from human platelets. J Biol Chem 262: 4098–4103.
46. Cohen T, Gitay-Goren H, Neufeld G, Levi BZ (1992): High levels of biologically vascular endothelial growth factor (VEGF) are produced by the baculovirus expression system. Growth Factors 7:131–138.
47. Aprelikova O, Pajusola K, Kaipainen A, Korhonen J, Barleon B, Connoly D, Weich HA, Alitalo K (submitted): The Flt4 tyrosine kinase gene encodes a vascular endothelial growth factor receptor. EMBO J.

48. Marcelle C, Eichmann A (1992): Molecular cloning of a family of protein kinase genes expressed in the avian embryo. Oncogene 7:2479–2487.
49. Gospodarowicz D, Abraham JA, Schilling J (1989): Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci USA 86:7311–7315.
50. Maglione D, Guerriero V, Viglietto G, Ferraro MG, Aprellikova O, Alitalo K, Del Vecchio S, Lei KJ, Chou JY, Persico MG (1991): Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene 8:925–931.
51. Sato Y, Rifkin DB (1988): Autocrine activities of basic fibroblast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J Cell Biol 107:1199–1205.
52. Weich HA, Iberg N, Klagsbrun M, Folkman J (1991): Transcriptional regulation of basic fibroblast growth factor gene expression in capillary endothelial cells. J Cell Physiol 47:158–164.
53. McCarthy SA, Kuzu I, Gatter KC, Bicknell R (1991): Heterogeneity of the endothelial cell and its role in organ preference of tumor metastasis. Trends Pharmacol Sci 12:462–467.
54. Rosnet O, Marchetto S, deLapeyriere, Birnbaum D (1991): Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGF/CSF1R family. Oncogene 6:1641–1650.
55. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR (1991): A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell–enriched populations. Cell 65:1143–1152.
56. Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jaye M, Schlessinger J (1990): Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J 9:2685–2692.
57. Finnerty H, Kelleher K, Morris GE, Bean K, Merberg DM, Kriz R, Morris JC, Sookdeo H, Turner KJ, Wood CR (1993): Molecular cloning of murine FLT and FLT4. Oncogene 8:2293–2298.