The human VE-cadherin promoter is subjected to organ-specific regulation and is activated in tumour angiogenesis

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Running title: Regulation of the VE-cadherin promoter in organs and tumours

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ABSTRACT

Vascular endothelial (VE)-cadherin is exclusively expressed at interendothelial junctions of normal and tumour vessels. In this report, we characterised the transcriptional activity of the human VE-cadherin promoter. Transient transfection assays revealed that sequences at positions −1135/-744 and −166/-5 base pairs are critical for promoter activity in endothelial cells. We show that specific sequences in the proximal region interact with Ets and Sp1 family members. Transgenic mice were created and the human VE-cadherin promoter was able to confer correct temporal and spatial expression on the LacZ gene in embryos. In adults, the transgene was specifically and strongly expressed in lung, heart, ovary, spleen and kidney glomeruli, whereas expression was weak or absent in the vasculature of other organs, including brain, thymus, liver and skeletal muscle. Neovessels in tumour grafts and Matrigel implants harboured strong stainings, indicating that promoter activity is enhanced in angiogenic situations. Furthermore, Matrigel and transfection assays showed that VE-cadherin promoter is subjected to bFGF induction. Transgene expression was also noticed in extravascular sites of the central nervous system, suggesting that silencer elements may be located elsewhere in the gene. These results are a first step towards addressing the organ- and tumour-specific regulation of the VE-cadherin gene.

INTRODUCTION

Vascular endothelial-cadherin (VE-cadherin, CD144) belongs to the cadherin family of adhesive receptors (Breviario *et al.*, 1995; Gumbiner, 1996; Lampugnani *et al.*, 1992). This molecule was shown to play a crucial role in vessel assembly (Carmeliet *et al.*, 1999; Corada *et al.*, 1999; Corada *et al.*, 2002; Gory-Fauré *et al.*, 1999; Liao *et al.*, 2002) and endothelial permeability (Corada *et al.*, 2001; Gao *et al.*, 2000; Gotsch *et al.*, 1997; Gulino *et al.*, 1998; Hordijk *et al.*, 1999; Wong *et al.*, 1999). VE-cadherin is specifically located at interendothelial junctions (Breier *et al.*, 1996; Lampugnani *et al.*, 1992) and at the surface of angioblasts (Asahara *et al.*, 1999; Gill *et al.*, 2001; Nishikawa *et al.*, 1998). This transmembrane protein is a universal marker of all types of endothelia and its expression was detected as early as embryonic day (E)7.5 in angioblasts of mouse embryos (Breier *et al.*, 1996; Lampugnani *et al.*, 1992).

The endothelial-specific transcriptional activity of VE-cadherin could be located within a 2.5 kb sequence upstream of the mouse gene (Gory *et al.*, 1999). This region contains Ets and Sp1 binding sites essential for its activity (Gory *et al.*, 1998). Recently, we used this promoter to drive herpes virus-thymidine kinase gene expression in the endothelium of transgenic mice (Dancer *et al.*, 2003). Thymidine kinase immunohistological data revealed that transgene expression was high in tumour vasculature but weak in normal tissues, except in the endocardium. Accordingly, injection of the thymidine kinase substrate ganciclovir could inhibit growth of experimental tumours by limiting vessel density. Altogether, these features demonstrate the potential utility of the VE-cadherin promoter to target angiogenic endothelium.

In view of using the VE-cadherin promoter to target therapeutic genes in humans, we characterised the regulatory sequences of the human gene in vitro and in vivo. Our data highlight the existence of an organ-specific regulation of a constitutive endothelial gene. Furthermore, our results indicate species similarities and differences in the transcriptional control of VE-cadherin gene and point to the potential limitations of using cell type-specific promoters across species, for instance in gene

therapy strategies. Therefore, the hVE-cadherin promoter may represent a more relevant tool for applications in humans.

RESULTS

Sequence analysis

A 4.8 kb Xho I-fragment of the 5´-region of the human VE-cadherin gene was cloned from a human BAC-library. Database searches performed with the fully sequenced fragment revealed that the VE-cadherin promoter sequence is located on chromosome 16 (Direct Submission Production Sequencing Facility, DOE Joint Genome Institute, Walnut Creek, CA accession number: AC012325.7).

Sequence analysis further revealed that, comparable to the mouse gene (Huber *et al.*, 1996), the 5´-region of the human VE-cadherin gene is interrupted by an intron 30 bp upstream of the ATG codon (Fig. 1A). The 3´-Xho I site of the cloned fragment is located within the first intron, 135 bp downstream of the splice donor site.

The transcription start site was localised by primer extension. An oligonucleotide homologous to the noncoding strand of the cDNA sequence (positions –37/-68 from initiation codon), was end-labelled, annealed to total human placenta RNA and incubated with reverse transcriptase. Extended products were analysed by electrophoresis in denaturing 6% polyacrylamide gel (Fig. 1B). The major band was 57 nucleotide long. This size is consistent with a transcription start site at a C located at position –93 upstream of the ATG in the cDNA sequence. As transcription preferentially initiates at a A, it is likely that the A at position –94 be the real transcription initiation site. This site will be designated as +1 in the promoter sequence.

In vitro analysis of the VE-cadherin promoter

In order to identify promoter sequences conferring endothelial-specific reporter gene expression, hVE-cadherin promoter fragments from positions –3499 to –166 at the 5'-end to position -5 bp at the 3'-end, were cloned into a luciferase reporter gene plasmid.

Reporter gene expression conferred by these promoter fragments was analysed by transient transfection experiments of human umbilical vein endothelial cells (HUVEC), bovine aortic endothelial cells (BAEC) and nonendothelial human cells (HeLa, HEK-293). An expression plasmid coding for the luciferase from *Renilla reniformis* was included in each experiment to normalise transfection efficiency. Compared to the promoterless pGL3basic vector, luciferase activities of the hVE-cadherin constructs were 33-100 (HUVEC) and 60-120 (BAEC)-fold higher in endothelial cells (Fig. 2). In contrast, in HeLa and HEK-293 cells, hVE-cadherin promoter activities were in the range of 2 -fold above background (Fig. 2). In HUVEC and in a lesser extent in BAEC, a decrease in reporter activity was observed when the –1135/-744 fragment was deleted, suggesting the existence of an enhancer element in this region. The shortest promoter fragment, -166/-5, retained endothelial cell-specificity. Interestingly, mouse and human VE-cadherin promoters yielded comparable reporter activity in BAEC and HUVEC (Fig.2).

Altogether, these data show that the isolated hVE-cadherin sequence harbours a promoter activity specific to endothelial cells. This activity is concentrated in two regions, the proximal 166 bp and the –1135/-744 domain.

Search for potential transcription factor binding sites in functional domains

Human and mouse VE-cadherin promoters share high homology in the proximal promoter regions, up to 900 bp upstream of the transcriptional start site. Sequence homology in the two functional domains identified by transfection experiments (Fig. 2) may represent conserved binding sites for transcription factors. In the –1135/-744 region, homology domains are located between positions –929 and –741 (Fig. 3A). To determine potential transcription factor binding sites an homology search with a transcription factor recognition sequence database (Transfac) was performed using MatInspector software (Quandt *et al.*, 1995). Database search showed that the sequence –908/-894 (underlined in Fig. 3A) represents a putative binding site for several zinc finger transcription factors. In the proximal region, four consensus Ets binding sites (noted EBS1-4) as well as a

putative Sp1 site are present (Fig. 3B). In the mouse sequence, two Ets binding sites (in bold) and the Sp1 site were shown to be essential for transcriptional activity (Gory *et al.*, 1998). Electrophoretic mobility shift assay using corresponding sites in the human sequence demonstrated a similar binding capacity to HUVEC nuclear factors (See Supplemental data). These data suggest that the Sp1 site as well as EBS 2 and 3 interact with specific transcription factors in human endothelial cells and thus participate in VE-cadherin gene transcription. Database search revealed consensus binding sites for zinc finger transcription factors (underlined in Fig. 3B). These CT boxes may represent endothelial zinc finger protein-2 (EZF-2) binding sites (Adachi & Tsujimoto, 2002). Indeed, these elements are in the vicinity of the Sp1 site, as previously described for the "scavenger receptor expressed by endothelial cell" (SREC) promoter (Adachi & Tsujimoto, 2002).

Interestingly, the 3.5 kb hVE-cadherin promoter contains neither Alu sequence, nor CpG island, whereas both of which are found within the mouse promoter (located at positions –1012/-811 and -1590/-1535, respectively). These types of elements are known to interfere with gene expression (Batzer & Deininger, 2002; Hewitt *et al.*, 1995; Shiraishi *et al.*, 2002; Turker, 2002). Although the activity of these elements have not been examined in the context of the mouse VE-cadherin promoter, their absence in the human promoter may have functional significance.

Generation of VEcad-LacZ transgenic mice

In vivo studies were performed with the 3.5 kb hVE-cadherin promoter fragment. The VEcad-LacZ expression construct was injected into fertilised mouse oocytes (CD1 strain). Newborns were genotyped by Southern blot using a LacZ probe, thereby identifying 6 transgenic founders. Southern blot analysis indicated that the six transgenic lines represent at least five independent insertion sites (not shown). Copy number (Table 1) was estimated by comparing intensity of the junction band (considered as one copy) with other bands using different Southern blots.

LacZ expression during embryonic development

Expression of LacZ was first screened by whole-mount X-gal staining of E12.5 embryos. Among the six transgenic lines, four harboured a blue colour in the vasculature as shown for line 31 in Fig. 4A and two a nonvascular expression (not shown). Data are summarised in Table 1. β−galactosidase expression of the four lines demonstrating vascular-specific X-gal staining was further examined by immunofluorescent labelling of parasagittal E12.5 embryo sections. β−galactosidase was present along the entire vasculature, as demonstrated by co-labelling with the endothelial marker CD31 (see Supplemental data). At higher magnification, co-distribution of β−galactosidase and CD31 could be noticed in the vasculature of all organs (not shown). At this stage, faint β−galactosidase signals were also detected in some regions of the brain parenchyma, suggesting that LacZ expression may not be fully restricted to the vasculature (not shown).

Earlier on, LacZ staining was detected in the yolk sac of E7.5 embryos (Fig. 4B), indicating that the hVE-cadherin promoter is active from the onset of vascular development and thereafter in the entire developing vasculature.

Vascular and nonvascular sites of LacZ expression in adult transgenic mice

Characterisation of LacZ expression in adult mice was performed by X-gal staining of tissues from three transgenic lines, 3, 31 and 55. The general expression pattern of the three mouse lines was similar, but expression levels were different, with line 31 harbouring the strongest stainings. Wild type controls did not show any labelling (not shown). Intense expression was observed in lung alveoli, but not in broncholi (Fig. 4C). Strong stainings were also noticed in the vasculature of spleen (Fig. 4D), ovary (Fig. 4E), oviduct (not shown) and kidney glomeruli (Fig. 4F, G); in contrast, extraglomerular vessels were rarely stained (Fig. 4F). In the heart, the endocardium was labelled as well as some myocardial vessels (Fig. 4H). X-gal labellings were weak in liver sinusoids and absent in other liver vessels (Fig. 4I). Some endothelial cells were stained in adrenal gland and skin (not shown). LacZ expression was absent in thymus, skeletal muscle and brain vasculature (not shown).

To examine promoter activity in tumour angiogenesis, transgene expression was assessed in the neovasculature of Lewis lung carcinoma implanted subcutaneously. In all examined tumours, we could find X-gal-stained vessels (Fig. 4J), with a higher density at the tumour periphery. This finding suggest that the VE-cadherin promoter may be re-activated by angiogenic stimuli produced by tumour cells. Tumour sections were co-immunolabelled with anti-β-galactosidase (Fig. 4K) and anti-CD31 (Fig. 4L) antibodies. Numerous CD31-positive vessels showed significant β-galactosidase labelling (arrowhead); however, some others were devoid of staining (arrow), indicating that transgene expression was not complete in the tumour vasculature. To determine which factor may trigger transgene expression, we performed a nontumoural angiogenesis assay. Likewise, Matrigel plugs supplemented with bFGF and VEGF were established and harvested at day 3 and 21, before and after vascular ingrowth, respectively. At day 3, intense X-gal stainings were observed in muscle vessels close to the plug (Fig. 4M). At day 21, LacZ expression was found in plug vessels (Fig. 4N). This feature suggests that promoter upregulation was not due to hypoxia but to VEGF or bFGF diffusing from the plug. To discriminate between the two cytokines, plug assays were performed with either VEGF or bFGF or none (n = 6 for each condition). The plugs were harvested at day 6, to allow embedding in connective tissues, even in the absence of bFGF. X-gal-positive plugs were only found when bFGF was added to the Matrigel. Co-labelling of bFGF-containing plug sections with X-gal (Fig. 4O) and anti-CD31 (Fig. 4P) showed that β-galactosidase was partially expressed in plug vessels. In VEGF-containing plugs, absence of X-gal staining (Fig. 4Q) could not be attributed to the lack of vascular ingrowth as CD 31-positive vessels were present (Fig. 4R). Interestingly, muscle capillaries in the periphery of VEGF-containing plugs were not stained by X-gal either (not shown). Altogether, these data demonstrate that bFGF is required to activate the VE-cadherin promoter in this angiogenesis model.

LacZ expression data in adult vasculature are summarised in Table 2. These data show that the human VE-cadherin promoter has the capacity to drive reporter gene expression in the vasculature of a number of adult organs. However, transgene expression did not extend to all vessels, which

contrasts with VE-cadherin expression (See Supplemental data). Stainings were observed in several quiescent (lung, kidney, heart, adrenal gland and skin), as well as angiogenic (ovary, oviduct, tumour and Matrigel plug) vessels. These data support the hypothesis of an organ-specific regulation of the VE-cadherin promoter. Moreover, it is noteworthy that promoter re-activation occurred in a simple angiogenic model, such as the Matrigel plug assay, in a bFGF-dependent manner.

To examine a possible regulation of the human VE-cadherin promoter by bFGF in vitro, HUVEC were transfected with the –3499/-5 promoter/luciferase construct in the presence of increasing concentrations of bFGF. As shown Fig. 5, a dose-dependent induction of promoter activity was observed between 0 and 10 ng/ml. Stimulation was 2.2-fold for 10 ng/ml of bFGF. These results thus confirm that the human VE-cadherin promoter is bFGF-sensitive.

Whereas the hVE-cadherin promoter demonstrates endothelial specificity and the correct temporal and spatial expression pattern during embryonic development as well as in most organs of adult animals, some extravascular expression was noted in the brain, namely in the medial habenular nucleus and in the cerebellum (not shown). Immunohistological analysis of mouse brain using anti-VE-cadherin antibody confirmed that none of these regions harbours an extravascular VE-cadherin localisation (not shown). Therefore, these latter data indicate an ectopic expression of the transgene, independently of its genome insertion site.

DISCUSSION

Endothelial cells play a crucial role in a number of physiological processes and are also implicated in several diseases, including tumour growth and metastasis (Carmeliet & Jain, 2000). The fact that endothelial cells display a morphological heterogeneity has been known for years but the extent of endothelium molecular diversity was only recently established (Cleaver & Melton, 2003; Pasqualini *et al.*, 2002; Rajotte *et al.*, 1998). Likewise, it has been possible to define vessel-, organ- and disease-specific markers. However, the transcriptional pathways at the origin of such diversity are unknown. In this study, we show that transcription of VE-cadherin, a protein considered as a uniform marker of all types of endothelia, is in fact differentially regulated along the vascular tree and is activated in tumour-induced angiogenesis. It is therefore conceivable that a functional dissection of this promoter may identify cis-active elements that specifically target vasculature of tumours or individual organs.

The LacZ expression pattern of transgenic embryos is consistent with in situ hybridisation data of VE-cadherin mRNA (Breier *et al.*, 1996). β-galactosidase activity was notably present at the onset of VE-cadherin expression in the yolk sac of E7.5 embryos, thereby demonstrating a timed regulation of the promoter. Among the six transgenic lines harbouring the VE-cadherin-LacZ construct, four expressed the transgene in a similar expression pattern. This indicates that the tissue specificity of transgene expression is intrinsic to the hVE-cadherin promoter and relatively independent of the site of transgene integration.

As shown by histological data, LacZ was differentially expressed among the different organs in adult mice. β-galactosidase activity could not be detected in some tissues such as thymus, skeletal muscle and the brain vasculature, even after long incubation times. Furthermore, the hVE-cadherin promoter targeted particular regions of organs. For example, kidney glomeruli were strongly stained whereas β-galactosidase activity was barely detectable in the extraglomerular vasculature. Our data therefore are in agreement with the notion that endothelial cells are regulated by distinct phenotypic

and genetic programs. In Northern blot experiments, VE-cadherin mRNA levels in mouse organs are highly variable (Breier *et al.*, 1996). However, these types of studies do not allow the distinction as to whether differential mRNA levels are due to variations in the degree of vascularisation or to endothelial heterogeneity. Precise quantification in mouse tissues of endogenous VE-cadherin – mRNA and protein – per endothelial cell may bring further insights into this aspect of VE-cadherin regulation. It is, however, likely that elements preventing transcription extinction are missing in the transgene. Such additional regulatory sequences may for instance be located in upstream or intragenic regions. It is improbable that mis-interactions between mouse transcription factors and human cis-acting elements are responsible of transgene quenching as this would be similar in all endothelial cell types. Although endothelial heterogeneity along the vascular tree is well documented, little is known about the transcriptional elements controlling this diversity. Aird et al.(Aird *et al.*, 1997) previously reported that the von Willebrand factor was regulated by organ-specific cis-active elements. Interestingly, its vascular map of expression was different from that reported here, indicating that these two genes differ in their transcriptional regulation.

VE-cadherin is required for the extension of the primitive vasculature during embryonic angiogenesis (Gory-Fauré *et al.*, 1999), which is consistent with its role in membrane protrusion activation (Kouklis *et al.*, 2003). It is thus possible that a burst of VE-cadherin expression may be required to promote and sustain vascular outgrowth. Interestingly, β-galactosidase activity was particularly high in angiogenic situations, namely in ovaries, Matrigel plugs impregnated with bFGF, tumours and during development. Similarly, the mouse promoter showed strong activity in tumour endothelium (Dancer *et al.*, 2003). The fact that we observed angiogenic responsiveness with the hVE-cadherin promoter fragment in vivo is clearly of considerable therapeutic interest. The Matrigel assay and HUVEC transfection data indicate that the promoter fragment used in these experiments is responsive to bFGF. This cytokine can induce expression of several transcription factors in endothelial cells, such as EGR1, Sp1 and HoxD3 (Boudreau *et al.*, 1997; Khachigian & Collins, 1997; Kurz *et al.*, 2003). Furthermore, Ets transcription factors were recently shown to be

involved in bFGF-induced angiogenesis (Pourtier-Manzanedo *et al.*, 2003). Further work is necessary to determine which pathway is activated in bFGF-dependent VE-cadherin induction. However, it is reasonable to speculate that the tumour activation of the VE-cadherin promoter may also use bFGF as mediator.

The promoter fragment used in this study failed to restrict transgene expression to the vasculature. Indeed, some areas of the central nervous system were stained by X-gal, whereas nontransgenic brains were not, indicating that it is not attributable to unspecific staining. This represents ectopic expression, as VE-cadherin is exclusively located in the endothelium and there is no evidence that neural cells express VE-cadherin at a precursor stage. One possible explanation of this feature is that the 3.5 kb promoter sequence lacks silencer elements that control total transcription specificity, even in the human context. As previously noticed and as opposed to the mouse gene, the hVE-cadherin gene proximal region does not harbour an Alu sequence nor a CpG island, two elements known to attenuate gene expression (Batzer & Deininger, 2002; Hewitt *et al.*, 1995; Shiraishi *et al.*, 2002; Turker, 2002). Similar regulatory elements, or other types of silencers, may be located elsewhere in the human gene. Alternatively, another likely explanation is that transcriptional control in neural cells is different in man and mouse. For example, variations in transcriptional control across species may be caused by differential binding of transcription factors to Sp1 sites (Call & Wolfe, 2002; Palmer *et al.*, 2001; Zhao *et al.*, 2000). Examples of species-specific transcriptional regulation are numerous (Apostel *et al.*, 2002; Bronner *et al.*, 2002; Chen *et al.*, 2002; Peterkofsky *et al.*, 1999; Rehli, 2002; Shi *et al.*, 1999) and argue for the use of promoters in homologous systems. This becomes particularly evident for the VE-cadherin promoter as both the mouse (Gory *et al.*, 1999) and the human promoters (this study) have been analysed in transgenic mouse systems.

One of the inherent challenges of gene therapeutic interventions is to direct and limit the expression of exogenous genes to the site of disease. One way of achieving this is by using either tissue or cell type-specific promoters, such as by using endothelial-specific promoters, which may be useful under circumstances where one would like to either support or inhibit angiogenesis. The results

presented here support the potential usefulness of the hVE-cadherin promoter for gene therapeutic measures and emphasise the limitations of using an exogenous promoter across species.

The hVE-cadherin-LacZ mice described in this paper may also prove useful to isolate vascular or nonvascular populations of β-galactosidase-expressing cells using fluorescent sorting techniques (Fiering *et al.*, 1991; Nolan *et al.*, 1988). As there is no LacZ expression in the brain vasculature, isolation may also be applied at postnatal stages to cells at ectopic sites of the central nervous system, such as the habenular nucleus. Moreover, the hVE-cadherin promoter may be relevant for human applications in antitumour therapy.

MATERIALS AND METHODS

Cloning and analysis of the human VE-cadherin promoter, generation of plasmid constructs:

A human BAC-library was screened with a 2.5 kb fragment of the murine VE-cadherin promoter (Genbank accession Y 10887, (Gory *et al.*, 1999)). The screening was performed by Genome Systems Inc (St. Louis, MO). A 4.8 kb Xho I fragment of a positive BAC clone was subcloned into the Xho I site of pBluescript KSII (Stratagene) and both strands were sequenced (MWG-Biotech, Ebersberg). Alignment of human and mouse sequences was performed with the software developed by Smith and Waterman (Smith & Waterman, 1981). For determination of putative transcription factor binding sites, the Transfac database was screened with MatInspector software (Quandt *et al.*, 1995) at the site http://www.genomix.de. For promoter analyses, a 3.5 kb Sac I fragment was cloned into the Sac I site of pGL3basic (Promega) containing the luciferase gene as reporter. Several deletions of the original 3.5 kb promoter fragment were performed by digestion of the plasmid with Bam HI, Apa I, Msc I, Sma I and Pst I and religation of the vector to yield constructs –1409, -1135, -744, -405 and –166, respectively.

Primer extension

Total RNA were extracted from human term placenta with Trizol reagent. Thirty µg of placental RNA or yeast tRNA were incubated at 42°C for 16 h with 0.02 pmoles of ^{32}P end-labelled oligonucleotide derived from the first exon noncoding strand : 5'-TGTGGGCTGAGGGATGTTTCTGTTCCGTTGG-3' in 65% formamide, 0.56 M NaCl and 10 mM Tris pH 7. Then, reverse transcription was performed with Superscript reverse transcriptase. Products were electrophoresed in a 6% polyacrylamide gel containing 8 M urea, together with a radiolabelled marker consisting of PBR322 DNA digested with Hae III.

Cell Culture

BAEC were prepared as previously described (Gory *et al.*, 1999). HEK-293 and HeLa cells were obtained from the American Type Culture Collection and H5V cells were a generous gift from Dr Vecchi (Garlanda *et al.*, 1994). HUVEC were isolated from fresh umbilical cords, using collagenase type IA as described (Gimbrone *et al.*, 1978). Cells were cultured as previously described.

Transient transfections and luciferase measurements

Transient transfections were performed in triplicates. At least two independent experiments were done for nonendothelial cells and at least four for HUVEC and BAEC. pGL3basic vector without promoter was used as mock control. Endothelial cells were transfected with ExGen 500 (Euromedex) according to the manufacturer's protocol. For bFGF activation, the cytokine was included in the medium 24 h before harvesting. Nonendothelial cells were transfected with Lipofectamin (Invitrogen). The cells were harvested 48 h after transfection and luciferase activities were measured using the Dual-Luciferase Assay System from Promega in a luminometer (Lumat LB 9507, EG&G). Reporter luciferase values were normalised to the luciferase activity of the internal control.

Generation and Southern blot analysis of VEcad-LacZ transgenic mice

For the generation of transgenic mice expressing the LacZ gene under control of the VE-cadherin promoter, a plasmid was created containing the 3.5 kb VE-cadherin promoter fragment, the SV40 splice donor and acceptor sequence from plasmid CMVβ (Clontech) and LacZ gene. Briefly, 3.5 kb of the VE-cadherin promoter fragment were cloned as FspI/XhoI-fragment into the sites EcoRV/XhoI of plasmid ΔE1B (Microbix, Toronto, Canada). Subsequently the SV40 splice donor/acceptor and LacZ cassette from CMVβ was inserted as Xho I/Sal I fragment. The transgene liberated from plasmidic sequences was used for transgenesis (Memorec, Cologne, Germany) in

mice with CD1 genetic background. Genotyping was performed by Southern blot with LacZ probe. Transgenic lines were maintained heterozygous by backcrossing with wild type CD1.

β–galactosidase enzymatic staining of embryos and tissues

Embryos or adult organs were harvested and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-gal) using established procedures. Adult tissues were embedded in paraffin, sectioned at 5 μm and counterstained with eosin or nuclear fast red (both from Sigma), as indicated.

Implantation of Matrigel plugs

The Matrigel plug assay was performed essentially as described (Passaniti *et al.*, 1992), with some modifications. Anaesthetised mice were injected subcutaneously with 0.25 ml of Matrigel (Becton Dickinson), supplemented with either 125 ng of bFGF or 12.5 ng of VEGF or both (both from Pepro Tech Inc.). Mice were sacrificed at day 3, 6 or 21 post-implantation as indicated, and plugs were harvested and processed as described for X-gal staining of adult tissues.

Tumour model

Lewis lung carcinoma cells were established from a C57Bl6 background and therefore transgenic CD1/ C57Bl6 F1 hybrid mice were used to prevent tumour rejection. Lewis lung carcinoma cells were implanted as described (Dancer *et al.*, 2003). Mice were sacrificed when tumour volume reached 500 mm^3. Dissected tumours were processed for paraffin or Tissue-Tek embedding.

Immunolabelling

Tumour immunolabelling was performed on cryosections using rabbit anti-β-galactosidase (Rockland) and rat anti-CD31 (Vecchi *et al.*, 1994) antibodies, followed by cyanine 3-conjugated anti-rabbit IgG (Jackson Laboratories) and alexa 488-conjugated anti-rat IgG (Molecular Probes)

antibodies. Paraffin sections of X-gal-treated Matrigel plugs were stained with anti-CD31 as above after treatment with trypsin to unmask antigen, as previously described (Gory-Fauré *et al.*, 1999).

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Table 1: Endothelial-specific expression of LacZ and transgene copy number in VE-cadherin-LacZ transgenics

Line	3	13	31	51	55	66
Endothelial expression[a]	+	-	+	+	+	-
Estimated copy number[b]	2	2	6	3	>10	1

[a]Expression was assessed by whole-mount X-gal staining of E12.5 embryos. [b]Copy number was evaluated by comparing intensity between junction band and repeat bands in Southern blot.

Table 2: Vascular LacZ expression in adult tissues of transgenic mice

Tissues	Expression levels[a]		
	Line 3	Line 31	Line 55
Lung	+++	+++	++
Spleen	++	+++	+
Ovary	+++	+++	+++
Oviduct	+++	+++	+++
Kidney:			
Glomeruli	++	+++	++
Extraglomerular vessels	-	+	-
Heart			
Endocardium	+	++	+
Myocardial vessels	-	+	+
Liver[b]	+	+	-
Adrenal gland	-	+	-
Skin	+	+	-
Thymus	-	-	-
Brain	-	-	-

[a]Levels were estimated on sections after X-gal staining and classified as having high (+++), moderate (++) or low (+) levels of expression. Essentially absent signal is indicated as (-). [b]Sinusoids.

LEGENDS TO FIGURES

Figure 1: Physical map of the human VE-cadherin gene 5'-end and determination of the transcription start site

(A) An Xho I fragment, comprising approximately 4.6 kb of the 5´-flanking region, the first exon, and 135 bp of the first intron of the human VE-cadherin gene, was cloned. Xho I restriction sites are indicated by an X. The translation start site is located within the second exon (ATG). The 5'-end position of DNA fragments used in transfection (Fig. 2) and transgenic (Fig. 5) studies are shown. The dashed line symbolises a domain (900 bp) of homology with the mouse sequence. **(B)** The transcription initiation site was determined by primer extension. A radiolabelled oligonucleotide designed in exon 1 noncoding strand was annealed to human placenta RNA or yeast tRNA and extended with reverse transcriptase. Resulting products were electrophoresed in denaturing polyacrylamide gel. The autoradiography shows a characteristic ladder with a major band (arrow), 57 base long, corresponding to position –93 upstream of the first ATG codon.

Figure 2: In vitro analysis of human VE-cadherin promoter by transient transfection

5'-deleted fragments of the promoter (from positions indicated Fig. 1 to nucleotide -1), linked to the luciferase gene from *Photinus pyralis*, were transfected in endothelial (HUVEC and BAEC) and nonendothelial (HeLa, HEK-293) cells. In each assay, a *Renilla reniformis* luciferase expression plasmid was cotransfected and luciferase reporter values were normalised with control luciferase activity. "0" indicates the promoterless reporter vector. The hVE-cadherin promoter demonstrates transcriptional activity only in endothelial cells. The activity conferring cell-type specificity is located in the –166/-5 region in HUVEC and BAEC, as well as the –1135/-744 region in HUVEC. Transfection with the murine promoter (mVE-2248) allowed comparison of human and murine promoter transcriptional activities. Bars represent the mean +/- SEM of at least 2 (nonendothelial

cells) and 4 (endothelial cells) independent experiments performed in triplicates. * $p < 0.05$, ** $p < 0.001$ according to Student t-test.

Figure 3: Alignment of the proximal 5´-regions of the human and murine VE-cadherin genes

Nucleotide sequences shown are from the murine (accession: Y10887 / MMCDH5E1) and human 5´-regions of the VE-cadherin gene. Sequence alignment was performed by the algorithm of Smith and Waterman (Smith & Waterman, 1981). The figure shows the homology domains of two regions demonstrating transcriptional activity (-1135/-744 and –166/-5). Gaps are indicated by hyphens. The transcriptional initiation sites are designated as +1. First exon nucleotides are in bold. Putative recognition sites for zinc finger transcription factors (ZF), Ets family members (EBS) and Sp1 factor are underlined. The functional Ets and Sp1 sites in the murine sequence, conserved in the human sequence, are indicated in bold.

Figure 4: LacZ expression during embryonic development and in the vasculature of adult organs

Whole-mount X-gal stainings of embryos at E12.5 **(A)** and E7.5 **(B)** demonstrated LacZ expression in the entire vascular system of the embryos. In adults, X-gal stainings were observed in the alveoli, but not the broncholi, of the lung **(C)**, in the entire vasculature of the spleen **(D)** and the ovary **(E)**, in endothelial cells of kidney glomeruli (arrowheads in **(F, G)**),in some extraglomerular vessels (arrow in **(F)**), in endocardial cells (arrows) and in some myocardial vessels (arrowhead) of the heart **(H)**. Faint labelling was observed in liver sinusoids (arrowheads in **(I)**). Lewis lung carcinomas implanted subcutaneously **(J)** harboured numerous X-gal positive vessels (arrows). Immunofluorescent co-labelling of tumour sections with anti-β-galactosidase **(K)** and anti-CD31 **(L)** antibodies showed the presence of β-galactosidase-positive (arrowhead) and –negative (arrow) vessels. **(M)** Intense X-gal staining was located in vessels at the border of Matrigel plugs (containing VEGF and bFGF), at day 3 post-implantation (arrows), before vascular invasion

(trabeculae within the Matrigel are not formed by endothelial cells). At day 21 post-implantation **(N)**, X-gal staining was observed in neovessels of VEGF and bFGF-containing Matrigel (arrows). Co-labelling of bFGF-containing Matrigel with X-gal **(O)** and anti-CD31 **(P)** showed β-galactosidase-positive (arrowhead) and –negative (arrow) vessels. Co-labelling of VEGF-containing-Matrigel with X-gal **(Q)** and anti-CD31 **(R)** showed that all vessels were β-galactosidase-negative. Sections were counterstained with eosin **(C-J, M, N)** and hematoxylin **(J, M, N)**. b, bronchioli; g, glomerulus; m, Matrigel; ys, yolk sac. Bars: 1mm in **(A)**; 150 μm in **(B)**; 50 μm in **(C, G-I, K, L, O-R)**, 100 μm in **(D, J, M, N)** and 250 μm in **(E, F)**.

Figure 5: Activation of the human VE-cadherin promoter by bFGF

HUVEC were transfected by the hVE-cadherin promoter (-3499/-5)/luciferase construct in the presence of variable concentrations of bFGF, as indicated below the histogram. Bars represent the mean +/- SEM of 2 independent experiments performed in triplicates. Data show a dose-dependent induction of luciferase activity by bFGF. * $p < 0.001$.