

Role of vascular endothelial-cadherin in vascular morphogenesis

Sylvie Gory-Fauré¹, Marie-Hélène Prandini¹, Hervé Pointu², Valérie Roullot¹, Isabelle Pignot-Paintrand³, Muriel Vernet² and Philippe Huber^{1,*}

¹Laboratoire de Transgénèse et Différenciation Cellulaire, ²Atelier de Transgénèse, and ³Atelier de Microscopie Electronique, Département de Biologie Moléculaire et Structurale, INSERM IFR27, CEA-Grenoble, 17, rue des Martyrs, 38054 Grenoble, France

*Author for correspondence (e-mail: phuber@cea.fr)

Accepted 8 March; published on WWW 19 April 1999

SUMMARY

Vascular endothelial (VE)-cadherin is an adhesive transmembrane protein specifically expressed at interendothelial junctions. Its extracellular domain exhibits Ca²⁺-dependent homophilic reactivity, promoting cell-cell recognition. Mice deficient in VE-cadherin die at mid-gestation resulting from severe vascular defects. At the early phases of vascular development (E8.5) of VE-cadherin-deficient embryos, *in situ* differentiation of endothelial cells was delayed although their differentiation program appeared normal. Vascularization was defective in the anterior part of the embryo, while dorsal aortae and vitelline and umbilical arteries formed normally in the caudal part. At E9.25, organization of endothelial cells into

large vessels was incomplete and angiogenesis was impaired in mutant embryos. Defects were more severe in extraembryonic vasculature. Blood islands of the yolk sac and clusters of angioblasts in allantois failed to establish a capillary plexus and remained isolated. This was not due to defective cell-cell recognition as endothelial cells formed intercellular junctions, as shown by electron microscopy. These data indicate that VE-cadherin is dispensable for endothelial homophilic adhesion but is required for vascular morphogenesis.

Key words: Vasculogenesis, Angiogenesis, Cadherin, Gene targeting, Mouse

INTRODUCTION

During embryogenesis, the cardiovascular entity is the first system to become functional and its integrity is required for embryo development and survival (Risau, 1995). Endothelial cells differentiate from mesodermic progenitors and interconnect to form a primary vascular plexus. This process is called vasculogenesis and only occurs in visceral derivatives and somites (Pardanaud et al., 1996). The primary plexus is extended by angiogenesis, which involves sprouting of new vessels and various steps of remodelling, allowing the formation of a complex vascular network with vessels of different sizes. Within the embryo and allantois, the emergence of angioblasts (i.e. endothelial progenitors) leads to the formation of the main vascular trunks: primitive endocardium, dorsal aortae, cardinal veins, vitello-embryonic and umbilical vessels (Sabin, 1917; Hirako and Hiruma, 1981). Later, other vessels such as the intersomitic vessels and the capillary sprouts of the neural epithelium derive from the primitive vasculature. In the yolk sac, endothelial cells originate from hemangioblastic progenitors differentiating into blood islands, where hematopoietic cells are surrounded by an endothelial layer (Jolly, 1940; Choi et al., 1998). Vasculogenesis further proceeds by fusion of the blood islands that form a meshwork of blood-filled capillaries. When embryo and yolk sac vasculatures interconnect, the vitelline vascular plexus develops into a highly branched vascular tree (i.e.

angiogenesis) in the entire yolk sac, providing a respiratory system to the early embryo.

The description of these morphological changes has been further documented by the analysis of early endothelial markers. The sequential expression of endothelial-specific tyrosine kinase receptors defines successive differentiation steps (for a review on these receptors and their ligands, see Mustonen and Alitalo, 1995). Flk-1, one of the two receptors of vascular endothelial growth factor (VEGF), is the earliest known murine protein marking the commitment of the mesodermic cells to the endothelial lineage. It is followed by Tie-2 (the receptor of angiopoietin-1 and -2) and Flt-1 (the other VEGF receptor), and later by Tie-1 (Dumont et al., 1995). Targeted inactivation of these receptors and their ligands in transgenic mice leads to embryonic lethality due to differential defects in vasculogenesis or angiogenesis (Shalaby et al., 1995; Carmeliet et al., 1996a; Ferrara et al., 1996; Sato et al., 1995; Puri et al., 1995; Fong et al., 1995). Most of these knockouts revealed critical steps in vascular morphogenesis, especially in the establishment of vessels connections, emphasizing the role of cell-cell recognition.

In this paper, we evaluated the biological activity of vascular endothelial-cadherin (VE-cadherin) by targeted disruption in transgenic mice. VE-cadherin belongs to the cadherin family of adhesive transmembrane proteins promoting homotypic cell-cell interaction via their extracellular domain (Lampugnani et al., 1992). The cytoplasmic domain of cadherins is associated with

molecules called catenins including α -catenin, β -catenin and plakoglobin, which mediate the linkage of the cadherin cluster with the actin cytoskeleton (for a review, see Yap et al., 1997). VE-cadherin is exclusively and constitutively expressed at interendothelial junctions (Lampugnani et al., 1992). In mouse embryos, *VE-cadherin* transcripts have been detected as early as embryonic day 7.5 (E7.5) in the mesodermic aggregates from which blood islands originate (Breier et al., 1996). Evidence of VE-cadherin hemangioblastic expression was further substantiated by immunoreactivity of purified progenitors (Nishikawa et al., 1998). Later on, VE-cadherin was detected in all developing vessels as well as in the adult vasculature (Breier et al., 1996). Functionally, VE-cadherin is able to promote the assembly of the junctional complex and to develop homotypic adhesive reactivity (Lampugnani et al., 1995; Navarro et al., 1995). VE-cadherin participates in control of endothelial permeability to solutes and neutrophils (Lampugnani et al., 1992; DelMaschio et al., 1996; Allport et al., 1997). Furthermore, VE-cadherin expression has been shown to be downregulated in the disorganized cells of angiosarcomas, suggesting that this molecule may be involved in vascular morphogenesis (Martin-Padura et al., 1995). Role of cadherins in cell sorting and morphogenesis had been previously reported for E-cadherin, N-cadherin and R-cadherin (Takeichi, 1995).

In previous work, we showed that *VE-cadherin* disruption impaired vasculogenesis in embryonic stem (ES) cells-derived embryoid bodies (Vittet et al., 1997). Although endothelial cells differentiated normally from *VE-cadherin*^{-/-} ES cells, no vascular plexus formation could be observed within bodies. We report now that *VE-cadherin* homozygous null-mutation in transgenic mice is embryonic lethal due to severe vasculogenic defects in the yolk sac and embryo.

MATERIALS AND METHODS

Generation of mutant mice and progeny genotyping

The targeted vector was linearized by *NotI*, capped with hairpin-shaped oligonucleotides (Vittet et al., 1997) and electroporated into R1 ES cells, derived from 129Sv strain (Nagy et al., 1993). Transfectants were plated on mitomycin-treated neomycin-resistant primary embryonic fibroblasts as feeder layer and cultured in Dulbecco's modified Eagle's medium (GibcoBRL), supplemented with 15% fetal calf serum (Seromed) and 500 U/ml leukemia inhibitory factor (Esgro, GibcoBRL). 48 hours after transfection, 350 μ g/ml G-418 (Sigma) were added to the medium and were kept until aggregation with morulas. Selection with 2 μ M gancyclovir (Syntex) was performed from day 4 to day 8 post-electroporation. Resistant colonies were picked at day 10 and expanded for freezing and DNA analysis. Clones were screened for homologous recombination by Southern blot analysis using *NcoI* digests and a 3'-probe, external to the recombination fragment (Fig. 1A; Vittet et al., 1997). The blots were re-probed with the *neomycin resistance (neo^r)* gene to confirm recombination at the 5'-

end and to exclude other *neo^r* integration (data not shown). Targeted clones occurred at a frequency of ~12%. Two independent clones, called C6 and D2, were aggregated to OF1/Ico (Iffa Credo, L'Arbresle, France) blastomeres and transferred to foster mothers. Chimeras were mated to Balbc/Ico, and ES cells from both clones contributed to the germline. F₁ mice were bred with Balbc/Ico and subsequent matings were obtained by intercrosses, keeping the two mouse lines independent.

Genotyping of progeny was done by genomic polymerase chain reaction (PCR) from tail tip DNA (Hogan et al., 1994). For embryological studies, PCR were performed either from embryos, yolk sacs (Hogan et al., 1994) or from scraped sections (George et al., 1993). Primers for *VE-cadherin* exon 2 were: 5'-GGATGCA-

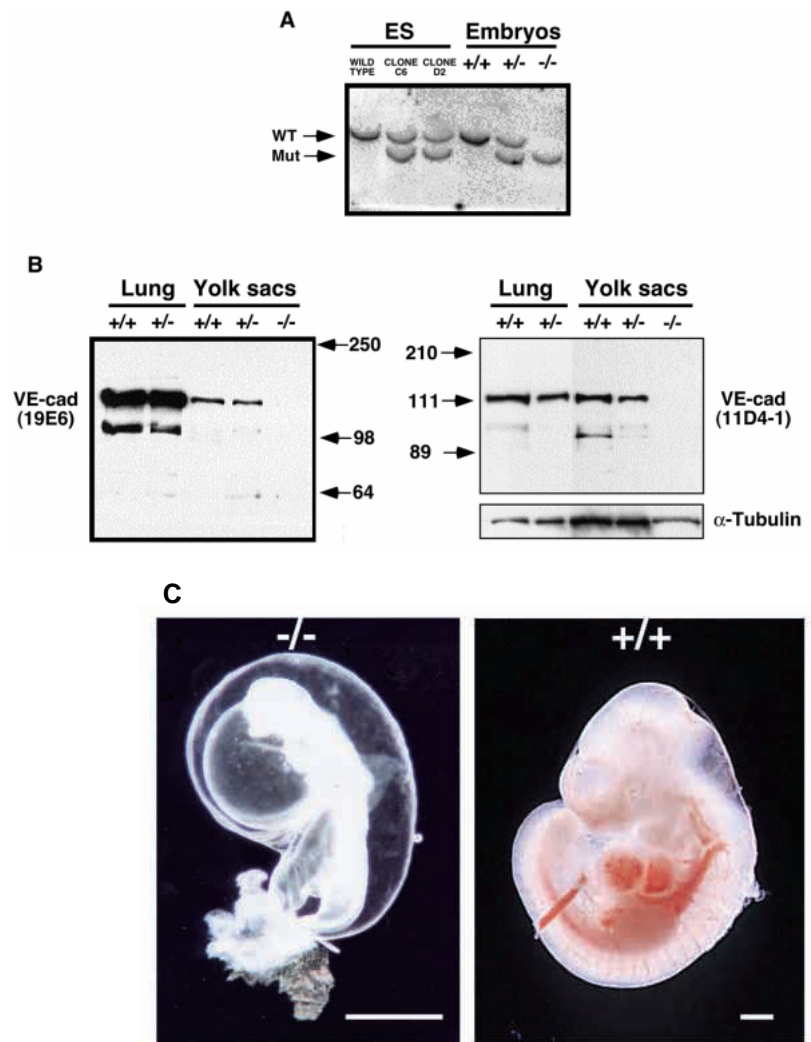


Fig. 1. Targeted disruption of *VE-cadherin* gene. (A) Southern blot analysis of targeted ES clones (C6 and D2) and offspring using *NcoI* digests and a 3'-external probe (Vittet et al., 1997), showing wild-type (WT) and mutated (Mut) loci. (B) Western blot analysis of adult lung and E9.5 yolk sac extracts of offspring from heterozygous intercrosses. Presence of VE-cadherin was revealed with two different antibodies: 19E6 (50 μ g of protein extracts per lane) and 11D4-1 (5 μ g or 25 μ g of lung or yolk sac extracts, respectively). Band at 100 kDa is a degradation product of VE-cadherin (111 kDa). Probing with a specific anti- α -tubulin antibody shows comparable protein amounts in the three yolk sacs. (C) External appearance of VE-cadherin-deficient (-/-) and wild-type (+/+) embryos at E10.5. At this age, the mutant embryo (still enveloped here by amniotic membrane) shows growth retardation, anemia, pericardial hypertrophy and incomplete folding. Scale bars, 400 μ m.

Table 1. Genotype analysis of *VE-cadherin*^{+/-} crosses

| Embryonic age | Number of litters | Genotype | | |
|---------------|-------------------|----------|-----|-----|
| | | +/+ | +/- | -/- |
| E9.0 | 2 | 3 | 11 | 2 |
| E9.5 | 7 | 20 | 33 | 16* |
| E10.5 | 7 | 17 | 33 | 15* |
| E11.5 | 2 | 8 | 8 | 3‡ |
| E12.5 | 2 | 6 | 12 | 0 |

*Macroscopically abnormal embryos.
‡Asystolic embryos.

GAGGCTCACAGAG-3' (5') and 5'-TCTCTTCATCGATGTGCATT-3' (3'). PCR products were electrophoresed, blotted and hybridized with ³²P-labelled internal probe: 5'-AGTTAGGGCCTGCCATTGCTGCCACTGCCA-3'. Primers for amplification of *neo*^r were: 5'-CCGACCTGTCCGGTGCCTTGAATGA-3' (5') and 5'-CGAGGAA-GCGGTACGCCATTCGCC-3' (3'). *neo*^r amplification products were visualized by ethidium bromide staining.

Western blot analysis

Lung from adult mice and E9.5 yolk sacs were lysed in 10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% Triton X-100, containing a protease inhibitory cocktail (Boehringer Mannheim) using a micro-potter (Kontes). Protein concentration was determined with the Biorad Protein Assay. Samples were analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membrane. VE-cadherin was detected with two different rat monoclonal antibodies directed against mouse VE-cadherin (clone 19E6; a generous gift from E. Dejana and clone 11D4.1, at 1 µg/ml, from Pharmingen) and visualized with horseradish peroxidase-conjugated anti-rat IgG (Jackson Laboratories; dilution 1:5000) and enhanced chemiluminescent detection reagents (Pierce). α -tubulin was detected with a rat monoclonal antibody (a generous gift from Dr Lafanechère).

Reverse transcriptase (RT)-PCR analysis

RT-PCR analyses were performed using exponential amplification conditions (Vittet et al., 1996). Oligonucleotides used as primers and probes for *VE-cadherin*, *platelet endothelial cell adhesion molecule (PECAM)-1* and *Flk-1* transcripts were previously described (Vittet et al., 1996). Primers for amplification of other transcripts and internal probes were: for *embryonic β -globin (β H1)*, 5'-CTCAAGGAGACCTTTGCTCA-3' (5'), 5'-AGTCCCCATGGAGTCAAAGA-3' (3'), 5'-GTCCAGGCTGCCTGGCAGAA-3' (probe); for *Tie-1*, 5'-TCTTTGCTGCTCCCCACTCT-3' (5'), 5'-ACACACACATTCGCCATCAT-3' (3'), 5'-CGCCAGCACCACCCACACAG-3' (probe); for *Tie-2*, 5'-CCTTCTACCTGCTACTTTA-3' (5'), 5'-CCACTACACCTTCTTTACA-3' (3'), 5'-CTGTTACCTCAGCCTTCAC-3' (probe); and for *Flt-1*, 5'-CTCTGATGGTGATCGTGG-3' (5'), 5'-CATGCCTCTGGCACTTG-3' (3'), 5'-AGAAGCCCCGCCTAGACA-3' (probe). *Hypoxanthine phosphoribosyltransferase (HPRT)* transcripts were co-amplified with each marker, using a primer set producing a short fragment (Vittet et al., 1996) for *Tie-1*, *Tie-2* and *Flt-1* amplifications, or a long fragment for the others in order to avoid co-migrating products. In the latter case, only the 5' oligonucleotide was different: 5'-AGTTCTTTGCTGACCTGCTG-3'. Reaction products were electrophoresed, transferred onto nylon membrane (Appligene) and hybridized with their respective probes and *HPRT* probe.

PECAM-1 and histological staining

For whole-mount PECAM-1 staining, embryos and yolk sacs were removed between E8.5 and E9.5, fixed 16 hours in 4%

paraformaldehyde/PBS at 4°C and stained with a rat monoclonal anti-mouse antibody (clone MEC 13.3, Vecchi et al., 1994), as described (Schlaeger et al., 1995). PECAM-1 immunoreactivity was revealed either with an alkaline phosphatase monoclonal anti-alkaline phosphatase procedure (Dako) in Fig. 6, or with an horse radish peroxidase-conjugated antibody (Jackson Laboratories) in Figs 3 and 4. For sections, embryos and yolk sacs were fixed in 1% paraformaldehyde/0.1 M phosphate buffer pH 7.4, for 16 hours at 4°C, rinsed in PBS, dehydrated through an ethanol series, cleared in xylene and paraffin embedded. Sections (7 µm) were used for hematoxylin and eosin staining or for PECAM-1 immunodetection. In this case, sections were treated with 1% trypsin (Sigma)/PBS for 30 minutes to unmask antigen. As secondary antibody, we used a cyanine 3-conjugated antibody (Jackson Laboratories) at 1:1000 dilution.

Electron microscopy

For electron microscopy, yolk sacs were fixed 1 hour in 2.5% glutaraldehyde/0.1 M sodium cacodylate at pH 7.2, rinsed three times in 0.1 M sodium cacodylate, then postfixed in 1% osmium tetroxide/0.1 M sodium cacodylate, dehydrated in graded ethanols, embedded in Epon and sectioned at 90 nm. Examination was performed on a Jeol 1200 ExII microscope at 80 kV.

Analysis of yolk sac hematopoiesis

Yolk sacs from E9.5 embryos were dissected and treated with 0.5% collagenase/0.1% DNase I/20% fetal calf serum in Dulbecco's modified Eagles medium for 1 hour at 37°C. After incubation, cells were dissociated by gentle flushing and counted. The total number of cells recovered per *VE-cadherin*^{-/-} yolk sac was ~60% that of the *VE-cadherin*^{+/+}. Cells were plated at a density of 3×10⁵ cells/ml in 0.9% methylcellulose/Iscove's modified Dulbecco's (GibcoBRL) medium

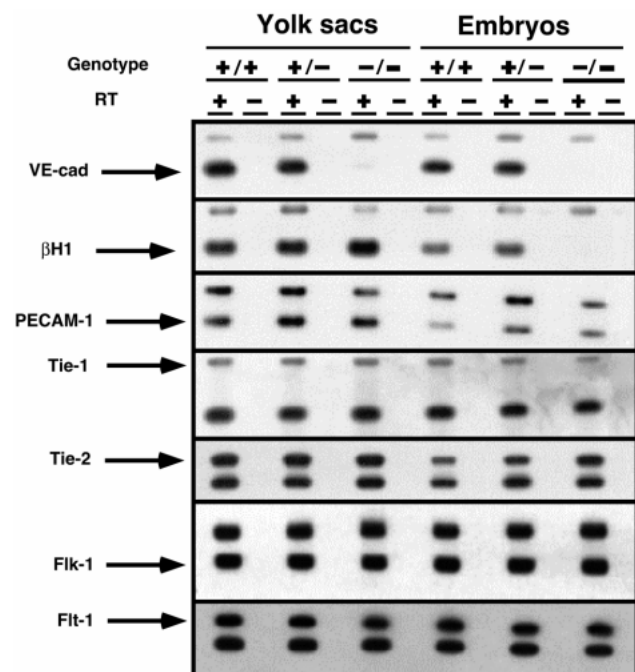


Fig. 2. RT-PCR analysis of E9.5 embryos (including allantois) and yolk sacs. Each marker RNA was co-amplified with *HPRT* transcript to evaluate cDNA variations between lanes. Each amplification was performed in presence (+) or in absence (-) of reverse transcriptase to detect genomic DNA contamination. One can note the loss of VE-cadherin signal in homozygous mice and the absence of β H1 mRNA within *VE-cadherin*^{-/-} embryos, indicating the lack of primary erythrocytes in the embryonic compartment.

containing factors for granulomonocytic-erythroid colonies formation: 50 ng/ml stem cell factor, 25 ng/ml interleukin-3, 25 ng/ml interleukin-6, 2 U/ml erythropoietin, 10 ng/ml granulocytic-colony stimulating factor (Preprotech) (Tronik-LeRoux et al., 1995). Colonies were scored by visual inspection after 7 days in culture.

RESULTS

Generation of mice heterozygous for the *VE-cadherin* null-mutation

The genomic structure of the mouse *VE-cadherin* gene was reported previously (Huber et al., 1996). In the targeted vector (already described in Vittet et al., 1997), a *neomycin phosphotransferase (neo^r)* expression cassette replaces part of exon 2, removing the initiation codon, the signal peptide, the propeptide and the first nine amino-acids of the mature protein. R1 ES cells were transfected with this construct and subjected to positive-negative selection (see Materials and Methods). Clones targeted at *VE-cadherin* locus were identified by Southern blot analysis (Fig. 1A). Homologous recombination was further confirmed by hybridization with the *neo^r* probe, which failed to detect any rearrangement of the targeted locus or additional sites of integration (data not shown). Two independent clones, C6 and D2, were aggregated to morulae and contributed to the germline of chimeric males. Genotyping of progeny was performed by Southern blot analysis (Fig. 1A) or PCR.

Mice lacking *VE-cadherin* die at mid-gestation

Heterozygous mice appeared phenotypically normal. Viability and fecundity were similar to the wild-type animals.

No homozygous pup was detected when heterozygous animals were intercrossed. Nevertheless, ratios between heterozygotes and wild types were normal: 114 versus 57 for clone C6, and 70 versus 35 for clone D2, respectively. These results indicate that *VE-cadherin* mutation is recessive embryonic lethal. To determine when homozygous embryos were dying, embryos were isolated at various stages of gestation (Table 1). Up to E10.5, wild-type, heterozygous and homozygous embryos were present. From E9.5, mutant embryos were clearly abnormal and were dead at E11.5. Both transgenic lines were analyzed in parallel and developed similar phenotypes. Defects included (Fig. 1C): growth retardation, reduced number of somites (by E10.5, somites number of mutant embryos never exceeded 20, as opposed to 35 in the wild type), incomplete turning, distended pericardial cavity, pallor and fragility. The heart beat until E10.5, but contractions were weak and irregular. No blood cell was visible within *VE-cadherin*^{-/-} embryos. Lack of embryonic β -globin (*BH1*) transcripts, as shown by RT-PCR analysis (Fig. 2), confirmed that primitive erythrocytes were absent from mutant embryos.

Loss of wild-type *VE-cadherin* gene expression

was established by RT-PCR analysis (Fig. 2). Furthermore, using two different monoclonal antibodies against mouse *VE-cadherin* (Fig. 1B), no signal could be detected in mutant yolk sacs (although endothelial cells were present: see below), whereas *VE-cadherin* was highly expressed in lung and yolk sac extracts of wild-type and heterozygous mice (Fig. 1B). Control experiment, using anti- α -tubulin antibody, shows that similar amounts of proteins were loaded in each lane (Fig. 1B). Thus, these data indicate that the targeted *VE-cadherin* mutation is a null allele.

Differentiation of endothelial cells

To see whether *VE-cadherin* loss of function interfered with endothelial differentiation, expression of endothelial markers

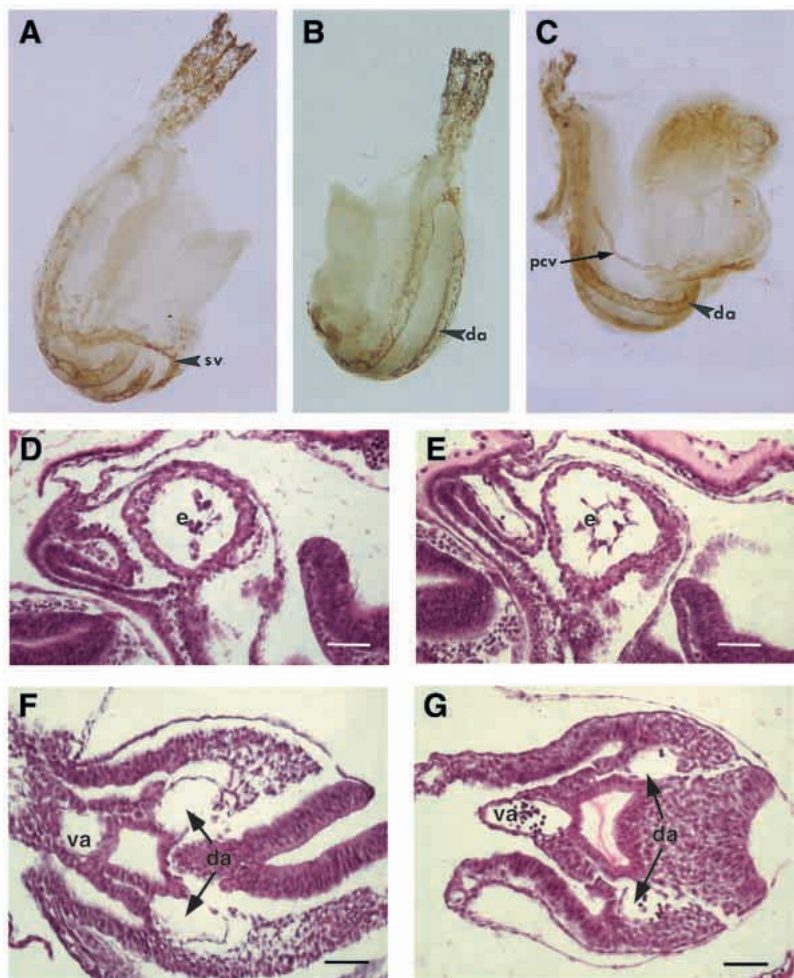


Fig. 3. Histological analysis of E8.5 embryos. (A-C) Whole-mount PECAM-1 staining. Genotyping of these embryos was not performed, but their phenotype unambiguously identified embryo in A,B as homozygote, and embryo in C as wild type or heterozygote. Note the absence of (i) PECAM-1-positive cells in anterior part of the mutant embryo (A) and (ii) posterior cardinal vein (B). Hematoxylin and eosin stainings of *VE-cadherin*^{-/-} (D,F) and *VE-cadherin*^{+/-} (E,G) embryo sections. At this stage, endothelial cells of endocardium were assembled in clusters in the homozygote (D) while the endocardium of the heterozygote possessed a lumen and formed trabeculae in the cardiac jelly (E). In the caudal part, the main vessels of the homozygote (F) appeared comparable to the wild type (G). Abbreviations: e, endocardium; da, dorsal aorta; pcv, posterior cardinal vein; sv, sinus venosus; va, vitelline artery. Scale bars, 50 μ m.

Table 2. Culture of yolk sac hematopoietic progenitors

| Genotype* | Number of colonies | | |
|-----------|--------------------|----|-------|
| | BFU-E | GM | mixed |
| +/+ | 11 | 5 | 11 |
| +/+ | 11 | 4 | 11 |
| -/- | 8 | 3 | 14 |
| -/- | 13 | 4 | 11 |

*Yolk sacs from the same litter.

Abbreviations: BFU-E, burst-forming unit-erythroid; GM, granulomonocytic.

was evaluated by RT-PCR with transcripts from E9.5 yolk sacs and embryos (including the major part of allantois). Signals obtained for *Flk-1*, *Flt-1*, *Tie-2*, *Tie-1* and *PECAM-1* (a uniform endothelial marker, although not restricted to the endothelial lineage; DeLisser et al., 1994) were similar for all three genotypes (Fig. 2). This suggests that endothelial differentiation could progress through all successive differentiation stages in intraembryonic and extraembryonic compartments of the mutants.

Vascular defects in mutant embryos

VE-cadherin^{-/-} embryos started to show extensive cell death at E9.5; by E10.5, the entire embryo had deteriorated (data not shown). Therefore, to investigate the causes of defective development of the mutants, we performed macroscopical and histological analyses before E9.5. Embryos were first examined by whole-mount staining with a monoclonal anti-PECAM-1 antibody to show the emerging vasculature (Fig. 3A-C). At E8.5 (8-10 somites), embryonic vascularization was consistently delayed in *VE-cadherin*^{-/-} embryos: (i) dorsal aortae in the caudal region were visible but appeared incompletely formed (Fig. 3B) in comparison to the wild type (Fig. 3C), (ii) sinus venosus was present but posterior cardinal veins were lacking (only exceptional vascular cords could be seen; Fig. 3A), while, in normal embryos, angioblasts amalgamated to form nearly continuous structures (Fig. 3C) and, most importantly (iii) no staining could ever be detected rostrally to the cardiac region (Fig. 3A,B), which was highly different from the wild-type or heterozygous littermates, for which a vascular network was obvious in the first aortic arch and in the head mesenchyme (Fig. 3C). On sections, endocardium was visible but endothelial cells failed to form a lumen (Fig. 3D), as opposed to the wild type (Fig. 3E). In the caudal part, no major difference could be seen between mutant and wild-type vasculatures except that mutant vessels were empty of blood cells (Fig. 3F,G). It is noteworthy that, in the mutant, vitelline artery was present on the embryonic side (Fig. 3F).

At E9.25, vascular deficiencies of *VE-cadherin*^{-/-} embryos were even more pronounced (Fig. 4A) and included: the presence of a delicate plexus rather than organized large vessels (Fig. 4B), especially in the mid-trunk region and limited angiogenic extensions such as the intersomitic vessels. Along the vitello-

embryonic stalk, endothelial cells assembled into vesicles (Fig. 4C) that did not form a continuous vessel, like in the heterozygous embryo (Fig. 4D). In the caudal region, both dorsal aortae and umbilical arteries started to show abnormal dilation between E9.25 and E9.5 (Fig. 4E). Development of the head microcirculation was partial (Fig. 4A) and PECAM-1 staining of sections revealed that organisation of angiogenic cords into vessels was not fully accomplished (compare Fig. 5A and B).

Extraembryonic compartments of *VE-cadherin*^{-/-} mutants harboured more severe phenotypes

Angioblasts appeared in *VE-cadherin*^{-/-} allantois, but instead of forming a dense vascular network, angioblasts aggregated and constituted a collection of islets (Fig. 4A). Furthermore,

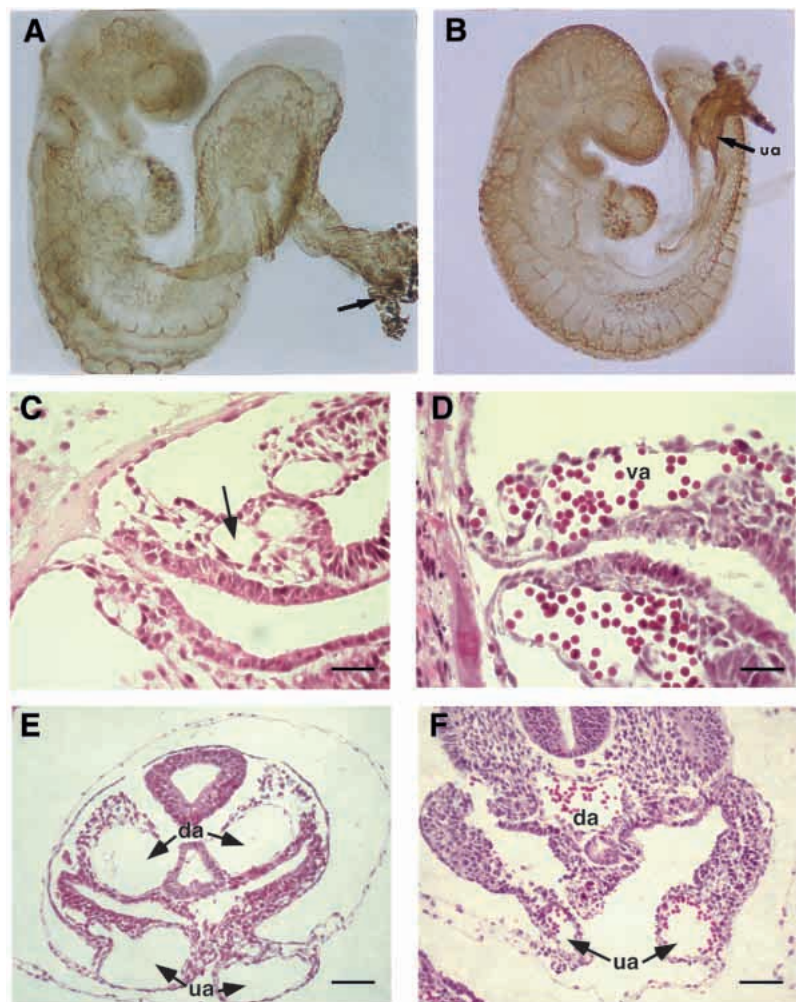


Fig. 4. Histological analysis of E9.25 embryos. Genotypes: (A,C,E) *VE-cadherin*^{-/-}; (B) *VE-cadherin*^{+/-} or *VE-cadherin*^{+/+}; (D) *VE-cadherin*^{+/-}, (F) *VE-cadherin*^{+/+}. (A,B) Whole-mount PECAM-1 staining. *VE-cadherin*-deficient embryos and allantois exhibited defective vascularization in both extension and organization (see text). Arrow in A shows endothelial clusters in *VE-cadherin*^{-/-} allantois. (C-F) Hematoxylin and eosin stainings of embryo sections. (C) Discontinuity of the vitello-embryonic vasculature (arrow). In the caudal part, mutant embryo showed dilation of dorsal aortae and umbilical arteries (E), when compared to the wild type (F). Abbreviations: da, dorsal aorta, ua, umbilical artery; va, vitelline artery. Scale bars, (A,B,E,F) 50 μ m; (C,D) 20 μ m.

umbilical vessels, although visible in the embryo (Fig. 5C,E), did not penetrate the allantois. Establishment of chorioallantoic junction occurred normally in the mutant and surprisingly, vascular organisation in the placenta was similar to the normal mice (Fig. 5G,H).

Blood islands differentiated normally from yolk sac mesoderm. However, endothelial cells failed to establish a primary vascular plexus, although some blood islands surrounding the placenta fused and formed cisternae instead of the vitelline arteries (Fig. 6A-D). It is noteworthy that blood-filled islands were concentrated in the placental hemisphere whereas blood cells were very few in the embryo-linked hemisphere where endothelial clusters were empty (Fig. 6B), even at the latest stages (E10.5). This feature had been previously reported for normal embryos, before fusion of blood islands (Jolly, 1940). Our study shows that not all the yolk sac has hematopoietic potentialities.

Lack of vessel assembly in *VE-cadherin*^{-/-} yolk sacs was confirmed by PECAM-1 whole-mount staining (Fig. 6F) (notwithstanding the presence of some angioblastic cords). In contrast, wild-type yolk sacs exhibited at this stage (E9.5) a honeycomb-like network in the process of remodelling (Fig. 6E). In the mutant, blood islands were constituted of blood cells densely packed by a closed endothelial layer (Fig. 5I). To see whether adherens junctions could form in the absence of VE-cadherin, blood islands were examined by electron microscopy. Mutant and wild-type yolk sacs showed similar pattern of electron-dense interendothelial junctions (Fig. 7), indicating that VE-cadherin is not required for endothelial homophilic adhesion. Interestingly, PECAM-1 was normally located at the junction, which could be better seen in intraembryonic vessels (Fig. 5E), thus confirming the establishment of large interendothelial contacts.

Altogether, these data suggest that VE-cadherin is dispensable for endothelial cells sorting and tissue cohesion but is required for vascular branching and morphogenesis.

Vitelline hematopoiesis is normal in *VE-cadherin*-deficient mice

As VE-cadherin is expressed by the hemangioblast, we reasoned that *VE-cadherin* gene disruption may also interfere with vitelline hematopoiesis. We thus examined the hematopoietic potential of the mutant yolk sac. E9.5 yolk sacs were dissociated by collagenase treatment and cells were cultured in semisolid medium, in the presence of factors for granulomonocytic-erythroid colonies formation (see Materials and Methods). The number of colonies of each type was similar in the mutants and in the wild type (Table 2), which indicates that primary hematopoiesis was not altered by VE-cadherin deficiency.

DISCUSSION

In this study, we have shown that VE-cadherin-deficient mice exhibited severe defects in both intraembryonic and extraembryonic vasculatures leading to lethal fetal wasting.

From E9.5, VE-cadherin-deficient embryos showed hypertrophy of pericardial cavity, as already described in other knockout embryos such as those lacking N-cadherin (Radice et al., 1997), Scl/Tal-1 (Shivdasani et al., 1995; Robb et al.,

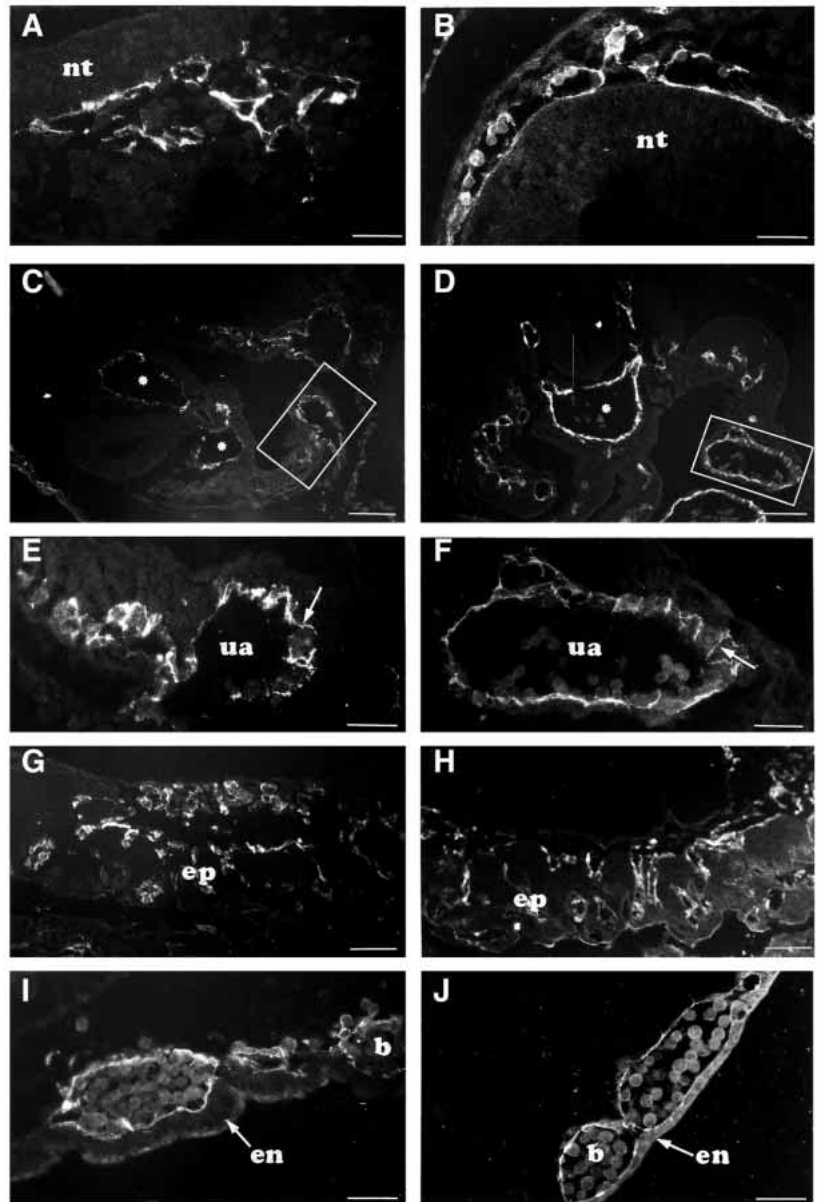


Fig. 5. PECAM-1 staining of E9.0 embryo sections. Genotypes are: (A,C,E,G,I) *VE-cadherin*^{-/-}; (B,D,F,H,J) *VE-cadherin*^{+/-}. The head capillary plexus of VE-cadherin-deficient embryos (A) was less elaborated than their heterozygous littermates (B). (C,D) Sections of the caudal region. (E,F) Higher magnifications of C,D, showing PECAM-1-positive interendothelial junctions of umbilical arteries (arrow). (G,H) Placentas of homozygotes and heterozygotes exhibited similar vascular extension. (I) Blood islands and (J) vitelline vessels of the yolk sac. Abbreviations: b, blood cells; en, yolk sac endoderm; ep, ectoplacental plate; nt, neural tube; ua, umbilical artery; *, dorsal aorta. Scale bars, (A,B,E,F,I,J) 300 µm; (C,D,G,H) 100 µm.

1995), vinculin (Xu et al., 1998) or tissue factor (Bugge et al., 1996), which is likely attributable to yolk sac deficiency. Dilation of distal aortae and umbilical arteries may also result from impossible flow delivery into both umbilical and vitelline vasculatures. These abnormalities, as well as extensive cell death at this gestation age, are probably due to abnormal extraembryonic vasculature and subsequent hypoxic conditions within embryo. However, at E8.5, at the time when embryonic and yolk sac circulations interconnect in the wild type, *VE-cadherin*^{-/-} embryos already exhibited defective vascularization, indicating a deficiency in intrinsic embryonic vasculature.

In embryo, in situ differentiation of endothelial cells was delayed, as revealed by absence of PECAM-1 staining in the anterior region. However, the differentiation program was not altered as all the markers tested were expressed. Later, organization into a primitive meshwork occurred almost normally, but coalescence of these capillaries or direct condensation of endothelial cells into large vessels was limited in the anterior and mid-trunk region, whereas dorsal aortae, umbilical and vitelline arteries appeared normal in the caudal part. Sprouting from dorsal aortae (i.e. intersomitic vessels) was also impaired. Altogether, these data support the notion that intraembryonic anomalies are related to both vasculogenesis and angiogenesis.

Phenotype was more drastic in yolk sac and allantois, where no capillary could form whether endothelial cells were associated with hematopoietic cells (in the placental pole of the yolk sac) or appeared alone (in the rest of the yolk sac and in allantois), than within embryos. These results indicate that VE-cadherin plays a critical role in vasculogenesis and more specifically in vascular branching of extraembryonic tissues. As pointed out by Hatzopoulos et al. (1998), distinct gene expression profiles exist in embryonic and extraembryonic vasculogenesis. This diversity may be due to different environments, such as perivascular cells or the surrounding matrix (Risau and Lemmon, 1988), or to independent endothelial lineages with their own specificities.

Closer observation of blood islands showed some angiogenic sprouts, but most of them failed to interconnect and remained isolated. When blood islands fused, they formed round cisternae, rather than a capillary plexus, as opposed to *VEGF*^{+/-} (Carmeliet et al., 1996a; Ferrara et al., 1996) and *tissue factor*^{-/-} (Bugge et al., 1996; Carmeliet et al., 1996b) yolk sacs for instance, for which a primitive plexus was visible. This clustering suggests that VE-cadherin participates in control of cytoskeleton organization and formation of elongated structures through intracellular signalling. Cross-talk between cadherins and integrins (transmembrane proteins involved in cell-matrix adhesion) was found to regulate cell mesenchymal properties and invasiveness of epithelial cells through β -catenin signalling pathway (Novak et al., 1998). Furthermore, interaction of cytosolic β -catenin with adenomatous polyposis coli protein (for a review,

see Ben-Ze'ev and Geiger, 1998) was shown to regulate epithelial tubulogenesis by promoting formation of long cell extensions, followed by assembly of cells into tubules (Pollack et al., 1997). VE-cadherin deficiency may thus alter endothelial plexus formation by modifying the β -catenin/adenomatous polyposis coli protein regulating pathway. Alternatively, a replacing molecule (see below) may promote stronger cell-cell adhesion (Huttenlocher et al., 1998), thus preventing migration and branching of endothelial cells, or may elicit other signal pathways.

In the yolk sac, blood cells were not spilled between endodermal and mesothelial layers, as would have been the case if endothelial cell-cell contacts were altered, but, on the contrary, they were densely packed by endothelial cells. Indeed, interendothelial junctions could be visualized by PECAM-1 staining and electron microscopy. Hence, other homophilic adhesive proteins may compensate for loss of VE-cadherin and provide sufficient strength to the junction for endothelial cohesion. Within mutant embryos, segregation of angioblasts from mesenchymal cells and their assembly indicate that other molecules allowed endothelial cell-to-cell recognition. Interestingly, N-cadherin, the other major cadherin expressed in human umbilical vein endothelial cells

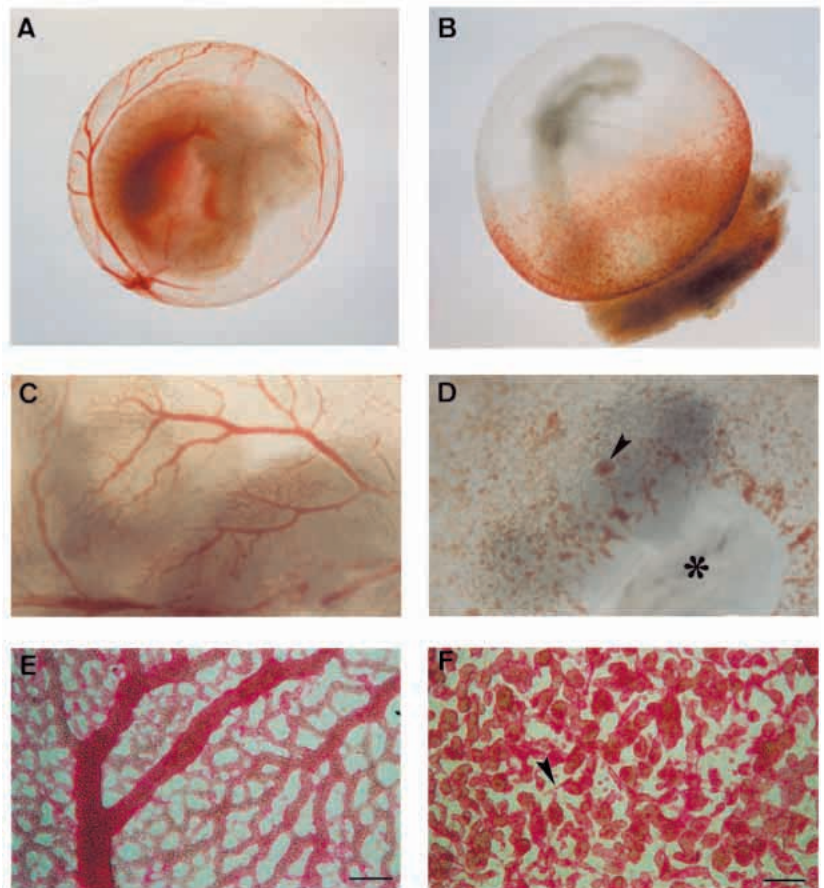


Fig. 6. External appearance of E10.5 yolk sacs (A-D) and whole-mount PECAM-1 staining of E9.5 yolk sacs (E,F). Blood islands of the homozygous mutants remained isolated (B,D,F), as opposed to the organized vasculature of the wild type (A,C,E). Blood-filled islands were distributed at the placental hemisphere (B). (D) Blood cisternae (arrow) and fusion of allantois to the chorion (*). (F) Arrow marks angiogenic extension. Scale bars, 100 μ m.

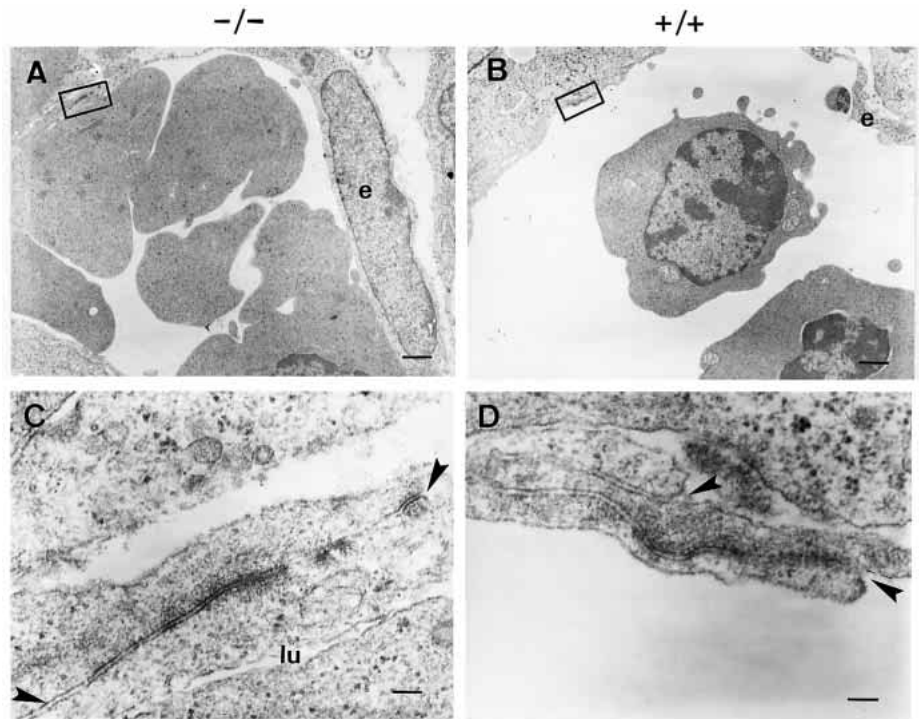


Fig. 7. Electron micrographs showing interendothelial junctions in the yolk sac. (C,D) Higher magnifications of A,B, respectively. The intercellular cleft (between arrows) contained comparable electron-dense junctions in the homozygote and in the wild type. Abbreviations: e, endothelial cell; Lu, lumen. Scale bars, A,B, 1 μ m; C,D, 100 nm.

(although not distributed at intercellular contacts), was shown to be excluded from the junction by VE-cadherin in a model of transfected Chinese ovary cells (Navarro et al., 1998). The possibility that N-cadherin could replace VE-cadherin at the junction, in VE-cadherin-deficient mice was tested by immunostaining with anti-N-cadherin antibody (13A9; a generous gift from M. Wheelock). Embryo and yolk sac histological sections did not show any reactivity with endothelia of homozygous and wild-type embryos (whereas the hindgut and the yolk sac endoderm were highly labelled; data not shown), suggesting that N-cadherin was not the molecule supporting endothelial cell-cell adhesion. These data confirm previous observations by Radice et al. (1997). Other homophilic adhesive molecules localized at endothelial junctions, such as PECAM-1 (Fawcett et al., 1995) or a recently identified proto-cadherin (i.e. a cadherin without catenin-binding domain) called vascular endothelial-cadherin-2 (Telo' et al., 1998), are potential candidates. It is possible that these proteins act as docking structures and promote endothelial cell-cell assembly (Fawcett et al., 1995).

The data obtained in this study are in agreement with the phenotype of VE-cadherin-deficient embryoid bodies (Vittet et al., 1997). In this *in vitro* model, VE-cadherin^{-/-} endothelial cells did not form a vascular plexus. Instead, endothelial cells remained dispersed with no elongated processes and failed to form aggregates of more than two or three cells, which parallels what we observed in mutant yolk sac and allantois. However, it is noteworthy that VE-cadherin^{+/-} embryoid bodies also showed some alterations in vasculogenesis, which was not observed in heterozygous mice. This difference may be due to lower VE-cadherin expression in ES-derived endothelial cells than in mice or to a distinct genetic background. In addition to these data, Bach et al. (1998) reported that anti-VE-cadherin blocking antibodies inhibited capillary tube formation of human umbilical vein endothelial

cells in fibrin or collagen gels, indicating that, in this model also, VE-cadherin is necessary for vascular architecture.

Lack of VE-cadherin did not significantly modify the quantity and distribution of yolk sac hematopoietic progenitors and their differentiation proceeded normally. Although it has been demonstrated that embryo (Dieterlen-Lièvre et al., 1997) and possibly the allantois (Downs and Harmann, 1997; Caprioli et al., 1998) contain hematopoietic stem cells at this age (E9.5), no circulating cell could be identified within embryonic vasculature and allantois clusters.

In conclusion, mice lacking VE-cadherin exhibit one of the most severe vascular phenotypes described in gene targeting experiments, after those for *Flk-1* and *VEGF* mutations. Furthermore, this study illustrates the morphogenetic role of cadherins, as opposed to a simply adhesive function.

We thank Professor Elisabeth Brambilla and Geneviève Chevalier for their help in embryo processing, Daniel Vittet for helpful discussion and Francis Aubouy for artworks. We also thank Drs. Andras Nagy, Reka Nagy and Wanda Abramow-Newerly for providing the mouse embryonic stem cell line R1. Animal experiments were conducted in agreement with the French and European Community guidelines for care of laboratory animals.

REFERENCES

- Allport, J. R., Ding, H., Collins, T., Gerritsen, M. E. and Luscinskas, F. W. (1997). Endothelial-dependent mechanisms regulate leukocyte transmigration: a process involving the proteasome and disruption of the vascular endothelial-cadherin complex at endothelial cell-to-cell junctions. *J. Exp. Med.* **186**, 517-527.
- Bach, T. L., Barsigian, C., Chalupowicz, D. G., Busler, D., Yaen, C. H., Grant, D. S. and Martinez, J. (1998). VE-cadherin mediates endothelial cell capillary tube formation in fibrin and collagen gels. *Exp. Cell Res.* **238**, 324-334.
- Ben-Ze'ev, A. and Geiger, B. (1998). Differential molecular interactions of β -catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Biol.* **10**, 629-639.

- Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnürch, H., Gotsch, U., Vestweber, D., Risau, W. and Dejana, E. (1996). Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* **87**, 630-642.
- Bugge, T. H., Xiao, Q., Kombrinck, K. W., Flick, M. J., Holmbäck, K., Danton, M. J. S., Colbert, M. C., Witte, D. P., Fujikawa, K., Davie, E. W. and Degen, J. L. (1996). Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. *Proc. Natl. Acad. Sci. USA* **93**, 6258-6263.
- Caprioli, A., Jaffredo, T., Gautier, R., Dubourg, C. and Dieterlen-Lièvre, F. (1998). Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. *Proc. Natl. Acad. Sci. USA* **95**, 1641-1646.
- Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. (1996a). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.
- Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., Müller, M., Risau, W., Edgington, T. and Collen, D. (1996b). Role of tissue factor in embryonic blood vessel development. *Nature* **383**, 73-75.
- Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.
- Del Maschio, A., Zanetti, A., Corada, M., Rival, Y., Ruco, L., Lampugnani, M.-G. and Dejana, E. (1996). Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *J. Cell Biol.* **135**, 497-510.
- DeLisser, H. M., Newman, P. J. and Albelda, S. M. (1994). Molecular and functional aspects of PECAM-1/CD31. *Immunol. Today* **15**, 490-495.
- Dieterlen-Lièvre, F., Cumano, A. and Godin, I. (1997). Cellules souches hématopoïétiques intraembryonnaires chez la souris: encore plus tôt. *Hématologie* **3**, 417-422.
- Downs, K. M. and Harmann, C. (1997). Developmental potency of the murine allantois. *Development* **124**, 2769-2780.
- Dumont, D. J., Fong, G.-H., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. (1995). Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev. Dyn.* **203**, 80-92.
- Fawcett, J., Buckley, C., Holness, C. L., Bird, I. N., Spragg, J. H., Saunders, J., Harris, A. and Simmons, D. L. (1995). Mapping and homotypic binding sites in CD31 and the role of CD31 adhesion in the formation of interendothelial cell contacts. *J. Cell Biol.* **128**, 1229-1241.
- Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.
- Fong, G.-H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70.
- George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. and Hynes, R. O. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**, 1079-1091.
- Hatzopoulos, A. K., Folkman, J., Vasile, E., Eiselen, G. K. and Rosenberg, R. D. (1998). Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development* **125**, 1457-1468.
- Hirakow, R. and Hiruma, T. (1981). Scanning electron microscopic study on the development of primitive blood vessels in chick embryos at the early somite-stage. *Anat. Embryol.* **163**, 299-306.
- Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo. A Laboratory Manual*. 2nd edition. Cold Spring Harbor, New York: Cold Spring Harbor Press.
- Huber, P., Dalmon, J., Engiles, J., Breviario, F., Gory, S., Siracusa, L. D., Buchberg, A. M. and Dejana, E. (1996). Genomic structure and chromosomal mapping of the mouse VE-cadherin gene (Cdh5). *Genomics* **32**, 21-28.
- Huttenlocher, A., Lakonishok, M., Kinder, M., Wu, S., Truong, T., Knudsen, K. A. and Horwitz, A. F. (1998). Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. *J. Cell Biol.* **141**, 515-526.
- Jolly, J. (1940). Recherche sur la formation du système vasculaire de l'embryon. *Arch. Anat. Microsci.* **35**, 295-361.
- Lampugnani M.-G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J. Cell Biol.* **118**, 1511-1522.
- Lampugnani, M.-G., Corada, M., Caveda, L., Breviario, F., Ayalon, O., Geiger, B. and Dejana, E. (1995). The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, β -catenin, and α -catenin with vascular endothelial cadherin (VE-cadherin). *J. Cell Biol.* **129**, 203-217.
- Martin-Padura, I., De Castellarnau, C., Uccini, S., Pilozzi, E., Natali, P. G., Nicotra, M. R., Ughi, F., Azzolini, C., Dejana, E. and Ruco, L. (1995). Expression of VE (vascular endothelial)-cadherin and other endothelial-specific markers in haemangiomas. *J. Pathol.* **175**, 51-57.
- Mustonen, T. and Alitalo, K. (1995). Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell Biol.* **129**, 895-898.
- Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424-8428.
- Navarro, P., Caveda, L., Breviario, F., Mântodeanu, I., Lampugnani, M.-G. and Dejana, E. (1995). Catenin-dependent and-independent functions of vascular endothelial cadherin. *J. Biol. Chem.* **270**, 30965-30972.
- Navarro, P., Ruco, L. and Dejana, E. (1998). Differential localization of VE- and N-cadherins in human endothelial cells: VE-cadherin competes with N-cadherin for junctional localization. *J. Cell Biol.* **140**, 1475-1484.
- Nishikawa, S.-I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. and Kodama, H. (1998). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hematopoietic lineages. *Development* **125**, 1747-1757.
- Novak, A., Hsu, S.-C., Leung-Hageteijn, C., Radeva, G., Papkoff, J., Montesano, R., Roskelley, C., Grosschedl, R. and Dehdar, S. (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β -catenin signalling pathways. *Proc. Natl. Acad. Sci. USA* **95**, 4374-4379.
- Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L.-M., Catala, M. and Dieterlen-Lièvre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122**, 1363-1371.
- Pollack, A. L., Barth, A. I. M., Altschuler, Y., Nelson, W. J. and Mostov, K. E. (1997). Dynamics of β -catenin interactions with APC protein regulate epithelial tubulogenesis. *J. Cell Biol.* **137**, 1651-1662.
- Puri, M. C., Rossant, J., Alitalo, K., Bernstein, A. and Partanen J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J.* **14**, 5884-5891.
- Radice, G. L., Rayburn, H., Matsunami, H., Knudsen, K. A., Takeichi, M. and Hynes, R. O. (1997). Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* **181**, 64-78.
- Risau, W. (1995). Differentiation of endothelium. *FASEB J.* **9**, 926-933.
- Risau, W. and Lemmon, V. (1988). Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Dev. Biol.* **125**, 441-450.
- Robb, L., Lyons, I., Li, R., Hartley, L., Köntgen, F., Harvey, R. P., Metcalf, D. and Begley, C. G. (1995). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc. Natl. Acad. Sci. USA* **92**, 7075-7079.
- Sabin, F. R. (1917). Origin and development of the primitive vessels of the chick and of the pig. *Contrib. Embryol. Carnegie Inst. Publ. (Wash.)* **6**, 61-124.
- Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70-74.
- Schlaeger, T. M., Qin, Y., Fujiwara, Y., Magram, J. and Sato, T. N. (1995). Vascular endothelial cell lineage-specific promoter in transgenic mice. *Development* **121**, 1089-1098.
- Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. L. and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62-66.
- Shivdasani, R. A., Mayer, E. L. and Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-434.
- Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619-627.
- Telo', P., Breviario, F., Huber, P., Panzeri, C. and Dejana, E. (1998). Identification of a novel cadherin (vascular endothelial cadherin-2) located at intercellular junctions in endothelial cells. *J. Biol. Chem.* **273**, 17565-17572.

- Tronik-Le Roux, D., Roullot, V., Schweitzer, A., Berthier, R. and Marguerie, G.** (1995). Suppression of erythro-megacaryocytopoiesis and the induction of reversible thrombocytopenia in mice transgenic for the thymidine kinase gene targeted by the platelet glycoprotein aIIb promoter. *J. Exp. Med.* **181**, 2141-2150.
- Vecchi, A., Garlanda, C., Lampugnani, M.-G., Renati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Ruco, L., Mantovani, A. and Dejana, E.** (1994). Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur. J. Cell Biol.* **63**, 247-254.
- Vittet, D., Buchou, T., Schweitzer, A., Dejana, E. and Huber, P.** (1997). Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc. Natl. Acad. Sci. USA* **94**, 6273-6278.
- Vittet, D., Prandini, M.-H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G. and Dejana E.** (1996). Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* **88**, 3424-3431.
- Xu, W., Baribault, H. and Adamson, E. D.** (1998). Vinculin knockout results in heart and brain defects during embryonic development. *Development* **125**, 327-337.
- Yap, A. S., Brieher, W. M. and Gumbiner, G. M.** (1997). Molecular and functional analysis of cadherin-based adherens junctions. *Annu. Rev. Cell. Dev. Biol.* **13**, 119-146.