View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Cell, Vol. 98, 147–157, July 23, 1999, Copyright ©1999 by Cell Press

Targeted Deficiency or Cytosolic Truncation of the *VE-cadherin* Gene in Mice Impairs VEGF-Mediated Endothelial Survival and Angiogenesis

Peter Carmeliet,[1,7] Maria-Grazia Lampugnani,[2] Lieve Moons,[1] Ferrucio Breviario,[2] Veerle Compernolle,[1] Françoise Bono,[3] Giovanna Balconi,[2] Raffaella Spagnuolo,[2] Bert Oosthuyse,[1] Mieke Dewerchin,[1] Adriana Zanetti,[2] Anne Angellilo,[1] Virginie Mattot,[1] Dieter Nuyens,[1] Esther Lutgens,[1] Frederic Clotman,[1] Marco C. de Ruiter,[4] Adriana Gittenberger-de Groot,[4] Rob Poelmann,[4] Florea Lupu,[5] Jean-Marc Herbert,[3] Désiré Collen,[1] and Elizabetta Dejana[2,6]

[1] The Center for Transgene Technology and Gene Therapy
Flanders Interuniversity Institute for Biotechnology
Leuven, B-3000, Belgium
[2] Mario Negri Institute for Pharmacological Research
20157 Milan, Italy
[3] Haemobiology Department
Sanofi, Toulouse, France
[4] Department of Anatomy and Embryology
Leiden University Medical Center
Leiden, The Netherlands
[5] Vascular Biology Laboratory
Weston Experimental Research Center
Thrombosis Research Institute
London SW3 6LR, United Kingdom
[6] Universita' dell' Insubria
Department of Clinical and Biological Sciences
School of Medicine
Varese, Italy

Summary

Vascular endothelial cadherin, VE-cadherin, mediates adhesion between endothelial cells and may affect vascular morphogenesis via intracellular signaling, but the nature of these signals remains unknown. Here, targeted inactivation (VEC$^{-/-}$) or truncation of the β-catenin-binding cytosolic domain (VEC$^{\delta C/\delta C}$) of the *VE-cadherin* gene was found not to affect assembly of endothelial cells in vascular plexi, but to impair their subsequent remodeling and maturation, causing lethality at 9.5 days of gestation. Deficiency or truncation of VE-cadherin induced endothelial apoptosis and abolished transmission of the endothelial survival signal by VEGF-A to Akt kinase and Bcl2 via reduced complex formation with VEGF receptor-2, β-catenin, and phosphoinositide 3 (PI3)-kinase. Thus, VE-cadherin/β-catenin signaling controls endothelial survival.

Introduction

Endothelial cells are linked to each other by tight- and adherens-type junctions. The latter are formed by transmembrane calcium-dependent adhesive proteins, called cadherins (Hynes, 1992; Kemler, 1992; Gumbiner, 1996). Endothelial cells contain VE-cadherin, only found at endothelial adherens junctions (Lampugnani et al., 1992), and N-cadherin, which is not clustered at cell–cell contacts (Salomon et al., 1992) and is also present in other cells (Kemler, 1992).

VE-cadherin interacts, via its cytoplasmic tail, with three proteins of the *armadillo* family, called β-catenin, plakoglobin, and p120. β-catenin and plakoglobin bind to α-catenin which, together with vinculin and α-actinin, anchors cadherins to the cortical actin cytoskeleton (Gumbiner, 1996; Nieset et al., 1997). The extracellular domain of VE-cadherin mediates initial cell adhesion, whereas the cytosolic tail is required for interaction with the cytoskeleton and junctional strength. VE-cadherin may also be implicated in cell differentiation, growth, and migration (Dejana, 1996). The extracellular adhesive domain and the intracellular β-catenin binding region of VE-cadherin participate in density-dependent inhibition of endothelial growth (Caveda et al., 1996).

Indirect evidence suggests that cadherins transfer intracellular signals but the intracellular mediators remain unknown. Cadherins may bind β-catenin and plakoglobin, thereby decreasing the cytoplasmic pool of free β-catenin. The latter can translocate to the nucleus and affect gene transcription via binding of high mobility group (HMG) transcription factors (Behrens et al., 1996; Nusse, 1997). Instead, β-catenin, bound to cadherins, cannot bind to HMG transcription factors (Fagotto et al., 1996). Cadherins may also participate in signaling by clustering signaling molecules and growth factor receptors. For example, the epithelial growth factor receptor can associate with the cadherin/catenin complex (Hoschuetzky et al., 1994) whereas several kinases and phosphatases codistribute with adherens junction components (Balsamo et al., 1996).

In the mouse, VE-cadherin is expressed in hemangioblasts from day 7.5 of gestation (E7.5) onward (Breier et al., 1996) and, thereafter, constitutively in all endothelial cells. Deficiency of VE-cadherin prevented organization of endothelial cells in vessel-like patterns in embryoid bodies (Vittet et al., 1997).

In order to define the role of VE-cadherin and of its binding to β-catenin in intracellular signaling, mice were generated that lacked a functional *VE-cadherin* gene (VEC$^{-/-}$), that expressed a mutant *VE-cadherin* gene lacking the β-catenin-binding cytoplasmic tail (VEC$^{\delta C/\delta C}$), or that did not express detectable VE-cadherin levels because of an intronic *neomycin phosphotransferase* (*neo*) gene (VEC$^{neo/neo}$). Surprisingly, all three strains died at E9.5 due to vascular insufficiency, caused by increased endothelial apoptosis. Deficiency or truncation of VE-cadherin blocked the capacity of endothelial cells to respond to survival signals induced by vascular endothelial growth factor type A (VEGF-A), by preventing formation of a complex consisting of VE-cadherin, β-catenin, phosphoinositide-3-OH kinase (PI3-K), and VEGF receptor-2 (VEGFR-2/Flk1/KDR). As a result, VEGF-A did not activate the serine/threonine protein kinase Akt (protein kinase B) and did not increase levels of Bcl2, both mediators of the antiapoptotic machinery (Nunez and del

[7] To whom correspondence should be addressed (e-mail: peter.carmeliet@med.kuleuven.ac.be).

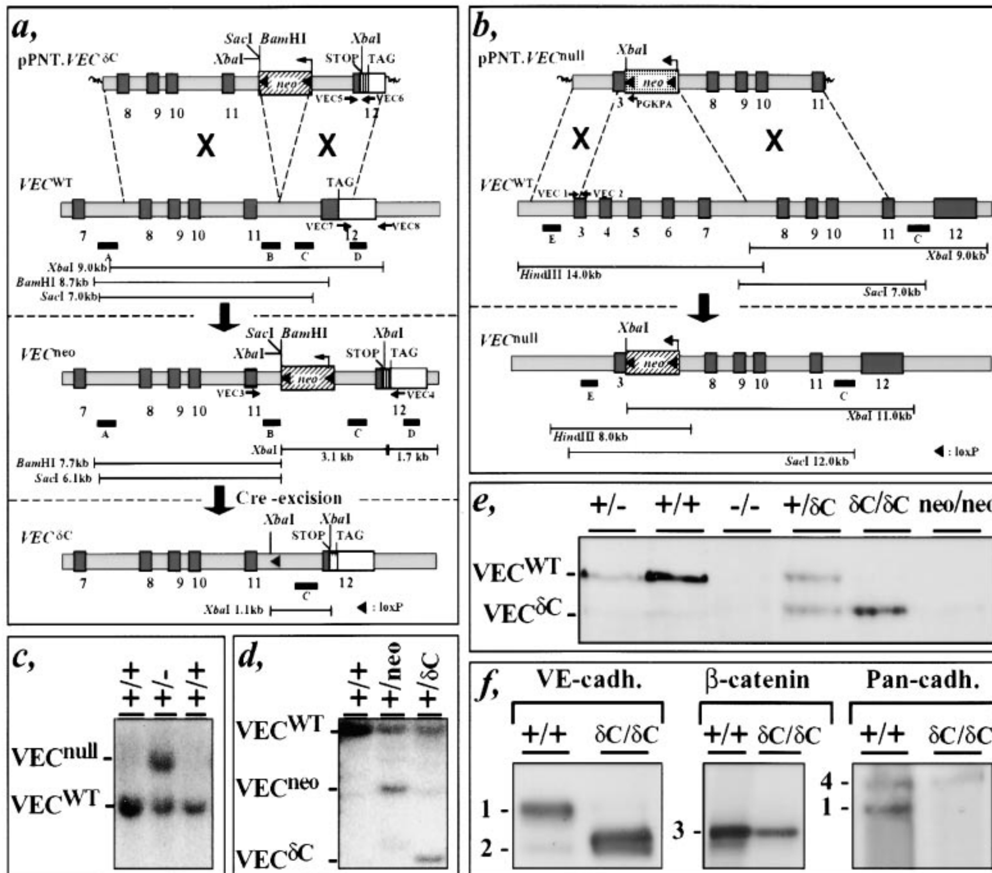

Figure 1. Targeting of the *VE-cadherin* Gene

(a) The truncation vector (pPNT.*VEC*$^{\delta C}$), the *VEC*^{WT} allele, the targeted allele containing a floxed intronic *neo*-cassette (*VEC*^{neo}) and the truncated (*VEC*$^{\delta C}$) allele are shown.

(b) The inactivation vector (pPNT.*VEC*^{null}), the wild type (*VEC*^{WT}), and the inactivated (*VEC*^{null}) allele are shown. Numbered exons (darkened blocks), introns (gray shaded bars), probes (lettered black boxes), restriction fragments (thin lines), novel restriction sites introduced by cloning, VEC-numbered PCR primers (arrows); STOP (termination codon introduced for truncation); TAG (endogenous termination codon of *VEC*^{WT}). The β-catenin binding domain in exon 12 is located between the STOP and TAG.

(c and d) Southern blot of SacI-digested genomic DNA hybridized with probe C, generating a 7 kb *VEC*^{WT} and a 12 kb *VEC*^{null} fragment (c), and of XbaI-digested DNA, hybridized with probe C, generating a 9 kb *VEC*^{WT}, a 3.1 kb *VEC*^{neo}, and a 1.1 kb *VEC*$^{\delta C}$ fragment (d).

(e and f) Western blotting of E9.0 embryos for VE-cadherin (e) or immortalized endothelial cells for VE-cadherin, β-catenin, and pancadherin (recognizing the cytoplasmic domain of VE-cadherin and N-cadherin) (f). Numbers in (f) indicate position of native VE-cadherin (1; $M_r \sim 116$ kDa), truncated VE-cadherin (2; $M_r \sim 97$ kDa), β-catenin (3; $M_r \sim 92$ kDa), and N-cadherin (4; $M_r \sim 135$ kDa).

Peso, 1998). Interestingly, VE-cadherin was required for the survival signal of VEGF-A, but not of basic fibroblast growth factor (bFGF). Thus, the present targeting study unveils a novel antiapoptotic pathway in endothelial cells, depending on intracellular signals via a pathway involving an endothelial adhesion molecule and a growth factor receptor.

Results

Targeted Inactivation and Truncation of the *VE-cadherin* Gene

Truncation of the *VE-cadherin* gene was achieved by introducing a stop codon in front of the 82 carboxy-terminal residues, interacting with β-catenin (Navarro et al., 1998), and by incorporating an intronic *neo* cassette, flanked by *lox*P sites (VEC$^{+/neo}$; Figure 1a). For inactivation of the *VE-cadherin* gene (VEC$^{+/-}$), exons 3 to 7 were deleted (Figure 1b). VEC$^{+/neo}$ ES cells were reelectroporated with Cre-recombinase to remove *neo* (VEC$^{+/\delta C}$). ES cells homozygous for the inactivated *VE-cadherin* allele (VEC$^{-/-}$), the truncated *VE-cadherin* allele containing *neo* (VEC$^{neo/neo}$), and the truncated *VE-cadherin* allele without *neo* (VEC$^{\delta C/\delta C}$) were obtained by high neomycin (G418) selection. Correct targeting was confirmed by Southern blotting (Figures 1c and 1d) and PCR analysis (not shown). Expression of VE-cadherin in embryos, in endothelial cells purified from differentiated ES cells, or in immortalized endothelial cells was comparable in VEC$^{+/+}$, VEC$^{+/\delta C}$, and VEC$^{\delta C/\delta C}$ cells, whereas VE-cadherin levels were ~50% of normal in VEC$^{+/-}$ and VEC$^{+/neo}$ cells and undetectable in VEC$^{-/-}$ and VEC$^{neo/neo}$ endothelial cells (Figures 1e and 1f). The undetectable expression in VEC$^{neo/neo}$ cells suggests that the intronic *neo* cassette aborted VE-cadherin expression.

Healthy VEC$^{+/neo}$, VEC$^{+/-}$, and VEC$^{+/\delta C}$ mice, the latter obtained by crossbreeding VEC$^{+/neo}$ mice with *pgk:cre*

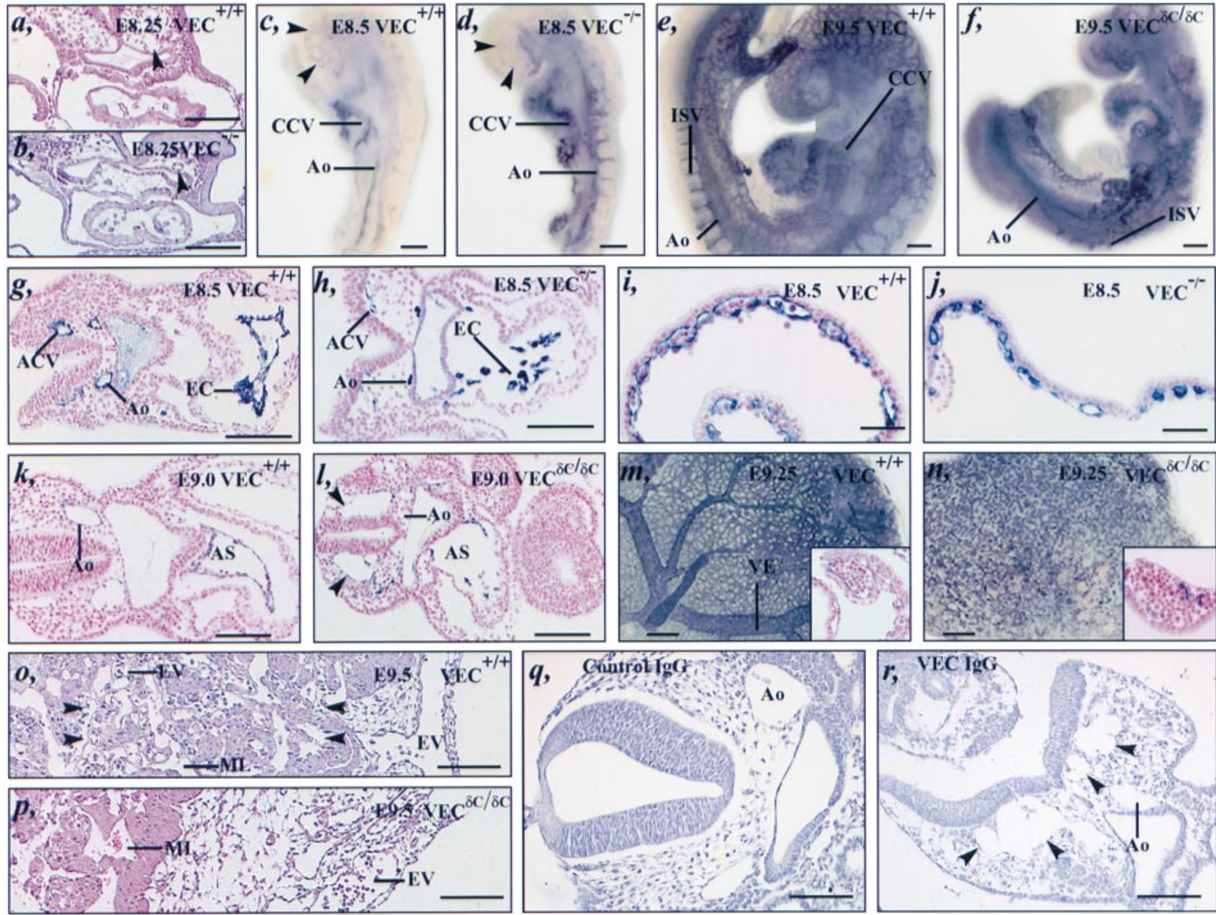

Figure 2. Abnormal Vascular Development in Mutant VE-Cadherin Embryos

(a and b) Hematoxylin-eosin staining of E8.25 $VEC^{+/+}$ (a) and $VEC^{-/-}$ (b) embryos, revealing assembly of endothelial cells in primitive vessels (arrowheads).

(c, d, and g–j) Vascular development at E8.5 (PECAM immunostaining). As compared to $VEC^{+/+}$, mutant embryos exhibit the following features: the aorta (Ao) and common cardinal vein (CCV) (c and d) and the yolk sac capillaries (i and j) are assembled, but the lumen in the thoracic aorta and anterior cardinal vein (ACV) is smaller (g and h); the cephalic vascular plexus contains irregular and dilated vessels (arrowheads in [c] and [d]); and the endocard (EC) is discontinuous and scrambled (g and h).

(e, f, and k–p) The vasculature ([e] and [f], JAM immunostaining; rest, PECAM immunostaining) in E9.0 and E9.25 $VEC^{+/+}$ embryos (e and k), yolk sacs (m), and placenta (o) matured into a branching network of large and small vessels with a well-formed aorta (Ao in [e] and [k]), common cardinal veins (CCV in [e]), sprouting intersomitic vessels (ISV in [e]), vitello-embryonic vessels (VE in [m]) and a placental labyrinth zone (between arrowheads in [o]) rich in embryonic vessels (EV in [o]) intermingled with maternal lacunae (ML in [o]), all containing an intact endothelial lining (inset in [m]). In contrast, the vasculature in mutant embryos (f and l), yolk sacs (n) and placenta (p) disintegrated, as revealed by the presence of only a few scrambled endothelial cells in the aortic sac (AS; panel [l]), the dilatation of the cephalic veins (arrowheads; panel [l]), the disappearance of endothelial cells in the thoracic dorsal aorta (Ao; panel [l]) or in vitelline vessels (inset in [n]), the loss of connections between yolk sac vessels at their branches (n), and the failure of embryonic vessels (EV) to invade deeply in the placenta (p).

(q–r) Injection of VE-cadherin antibodies in E8.5 embryos induced vascular defects (r) whereas vascular development was normal after injection of control IgGs (q).

Magnification bar is 100 μm in (a), (b), (g)–(l), and (o)–(r); and 200 μm in the other panels.

mice, were born at the expected frequency for Mendelian inheritance. Intercrossing of $VEC^{+/-}$, $VEC^{+/neo}$, or $VEC^{+/\delta C}$ mice did not yield viable $VEC^{-/-}$, $VEC^{neo/neo}$, or $VEC^{\delta C/\delta C}$ offspring, respectively, indicating that complete loss or homozygous expression of a truncated VE-cadherin induced embryonic lethality. Mutant embryos were recovered at E8.5 and E9.5 at the expected Mendelian frequency (25%, 24%, and 26% of 310 $VEC^{-/-}$, 450 $VEC^{neo/neo}$, and 280 $VEC^{\delta C/\delta C}$ embryos, respectively) but died at E9.5–E10. $VEC^{-/-}$, $VEC^{neo/neo}$, and $VEC^{\delta C/\delta C}$ embryos exhibited the same phenotype, described below. $VEC^{+/+}$ and mutant embryos initially contained a similar number of somites at E8.5 (Figures 2c and 2d), but by E9.0, mutant embryos became progressively growth retarded, contained smaller pharyngeal arches, and had fewer somites than $VEC^{+/+}$ embryos (11 $\pm$ 1.5 in $VEC^{-/-}$; 13 $\pm$ 0.8 in $VEC^{neo/neo}$; 14 $\pm$ 0.7 in $VEC^{\delta C/\delta C}$; 16 $\pm$ 0.4 in $VEC^{+/+}$; $p < 0.05$ $VEC^{+/+}$ versus others; Figures 2e and 2f). Mutant VE-cadherin embryos failed to progress beyond a developmental stage of E8.75–E9.0 and invariably died beyond E9.5.

Role of VE-Cadherin in Vascular Development

Histologic analysis and immunostaining for platelet endothelial cell adhesion molecule type-1 (PECAM/CD31) and junctional adhesion molecule (JAM) indicated that

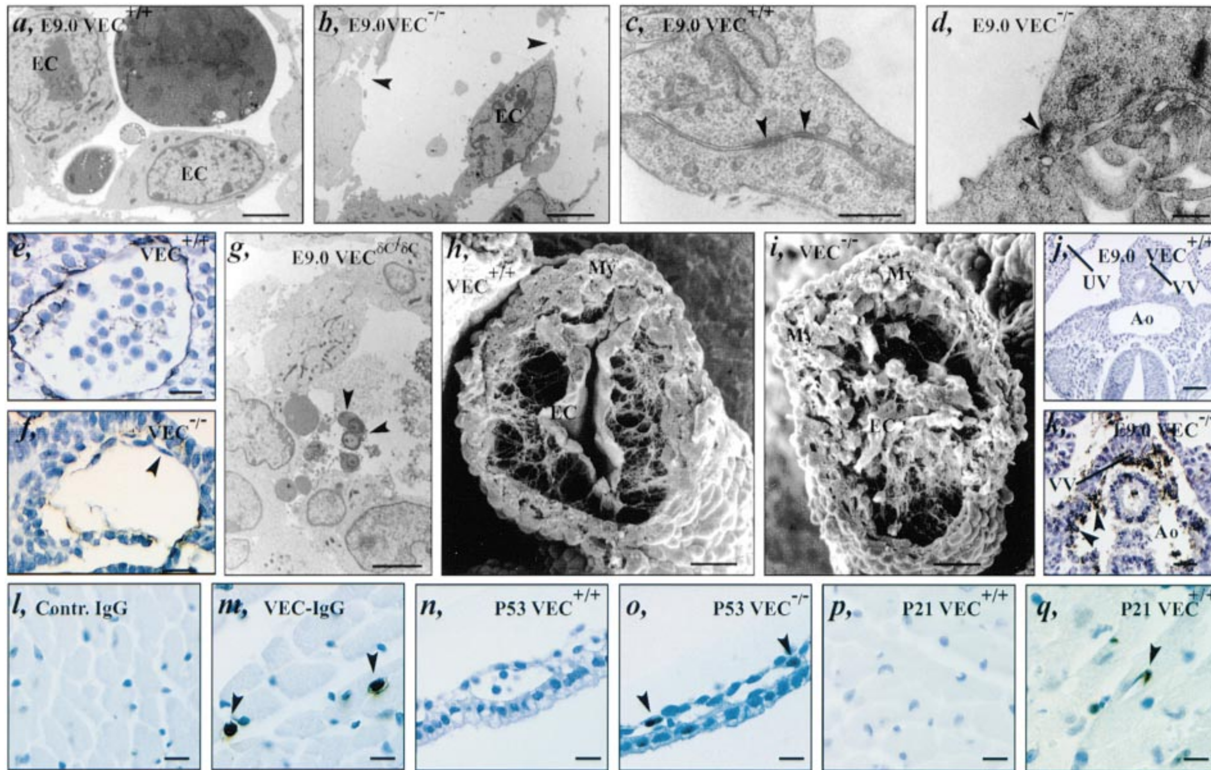

Figure 3. Endothelial Apoptosis in Mutant VE-Cadherin Embryos

(a–d) Transmission electron micrographs of E9.0 embryonic vessels, revealing an intact lining of endothelial cells (EC) in a VEC$^{+/+}$ vessel (a), in contrast to numerous gaps between mutant endothelial cells (arrowheads in [b]). VEC$^{+/+}$ endothelial cells adhered to each other via well-developed intercellular junctions over a long distance (arrowheads in [c]); junctional complexes were found between mutant cells, but less frequently and over short distances (arrowhead in [d]).

(e and f) Laminin immunostaining revealing firm attachment of endothelial cells in E9.0 VEC$^{+/+}$ vessels (e) and endothelial detachment from the basement membrane in VEC$^{-/-}$ embryos (f).

(g) Transmission electron micrograph of an apoptotic endothelial cell (arrowheads) in a mutant vessel.

(h and i) Scanning electron micrographs of the outflow tract of E8.75 embryos, revealing an intact and continuous endocard (EC) lining a ventricular cavity and a normal myocard (My) in VEC$^{+/+}$ embryos (h) but an endocardium that is detached, lies scattered in the center, occludes the ventricular lumen, and is surrounded by a loose and disorganized myocardium in mutant embryos (i).

(j and k) TUNEL staining, revealing apoptotic endothelial cells (arrowheads) in mutant (k) but not in VEC$^{+/+}$ (j) vessels. Ao, aorta; VV, vitelline vein; UV, umbilical vein.

(l and m) TUNEL-positive endothelial cells in hearts from adult wild-type mice are absent after injection of control antibodies (l) but numerous after injection of VE-cadherin antibodies (arrowheads in [m]).

(n and o) Nuclear p53 immunostaining was absent in wild type (n) but detectable in mutant yolk sacs (o).

(p and q) p21 immunostaining in myocardial endothelial cells was absent after injection of control antibodies (p) and detectable after injection of VE-cadherin antibodies (q).

Magnification bar is 1 μm in (c) and (d); 5 μm in (a) and (b); 10 μm in (g) and (l)–(q); 20 μm in (e), (f), and (k); and 50 μm in (h)–(j).

absent or truncated VE-cadherin did not prevent in situ differentiation of angioblasts to endothelial cells (Figure 2). In addition, mutant endothelial cells expressed comparable amounts of endothelial markers (see Supplemental Data below) and N-cadherin (Figure 1f), and produced laminin (Figures 3e and 3f), collagen IV, and the tight junctional molecule zona occludens (ZO)-1 (not shown). VEC$^{+/+}$ and mutant embryos were indistinguishable at E8.0, i.e., immediately after onset of angioblast formation (not shown). By E8.25–E8.5, angioblasts in both VEC$^{+/+}$ and mutant embryos differentiated to endothelial cells that became assembled in primitive vessels within the embryo and yolk sac (Figures 2a–2d), indicating that initial vasculogenesis occurred after loss/truncation of VE-cadherin. However, by E8.5, certain vessels in mutant embryos had a minimal or even no lumen (thoracic dorsal aorta, anterior cardinal vein; Figures 2g and 2h) whereas others were dilated (cephalic vessels). In mutant embryos, endothelial cells in the yolk sac formed channels that started to disconnect from each other at their branches (Figures 2i and 2j), whereas endocardial cells detached from each other and lay scattered in the ventricular cavity (Figures 2g and 2h). The lack of yolk sac–derived red blood cells inside E8.5 VEC$^{+/+}$ embryos indicated that the vitello–embryonic circulation was not yet established.

Vascular defects in mutant VE-cadherin embryos were more severe beyond E8.75–E9.0, when the primitive vascular network in VEC$^{+/+}$ embryos expands via sprouting angiogenesis and remodels in a branching network of large and small vessels. Endothelial cells throughout the entire vasculature progressively became disconnected

from each other and exhibited numerous gaps (Figures 3a and 3b), detached from their underlying basement membrane (Figures 3e and 3f), and lay scattered inside the lumen (Figures 2g, 2h, 2k, and 2l; endocardial cells in Figures 3h and 3i). Several of these endothelial cells were apoptotic, as evidenced by terminal deoxytransferase-mediated deoxyuridine nick end-labeling (TUNEL) (Figures 3j and 3k), annexin V staining, and transmission electron microscopy (Figure 3g). Sprouting of intersomitic vessels (Figures 2e and 2f) and remodeling into a network of large and small branches was impaired in mutant embryos and yolk sacs, often resulting in irregular, dilated vessels (cephalic vessels; Figures 2k and 2l). Other vessels were present as cords with a minimal lumen (pharyngeal arch vessels), contained few disconnected and scattered endothelial cells (outflow tract; Figures 2k and 2l; vitelline vessels; inset in Figures 2m and 2n), progressively disconnected from each other at branches, thereby inducing stagnating blood lakes (yolk sac; Figures 2m and 2n), or regressed (thoracic dorsal aorta; Figures 2k and 2l; vitelline and umbilical vessels). In $VEC^{+/+}$ placentas, allantoic vessels invaded the chorion by E8.5 and formed a labyrinth layer, in which embryonic vessels lay closely apposed to maternal lacunae by E9.5 (Figure 2o). In contrast, although mutant embryonic endothelial cells invaded the chorion, they failed to penetrate in the trophoblast layer and never established close interactions with the maternal sinuses (Figure 2p). As a result, mutant placentas only contained a spongiotrophoblast but not a labyrinth zone, remained much thinner (300 ± 40 μm in $VEC^{+/+}$ versus 110 ± 15 μm in $VEC^{-/-}$ at E9.5; $p < 0.05$), and did not develop beyond the E8.75 stage.

Beyond E9.25, vessels in mutant VE-cadherin embryos regressed, disintegrated, and collapsed (Figures 2e and 2f), causing circulatory insufficiency and progressive necrosis. Thus, normal VE-cadherin function is not or only minimally required for assembly into a primitive capillary plexus (vasculogenesis) but is essential for subsequent expansion, maturation, branching and remodeling into a network of veins and arteries of different sizes (angiogenesis), and prevention of vascular regression. A more detailed description of the mutant vasculature can be found in the Supplemental Data (see below).

Endothelial/Mural Cell Interactions in Mutant VE-Cadherin Embryos

Heart tubes in $VEC^{+/+}$ embryos looped at E8.75 and formed distinct chambers by E9.5, when the ventricular myocardium formed finger-like trabecular protrusions, lined by an intact endocardium. In mutant embryos, cardiac looping occurred but chamber development and trabeculation were impaired. Scanning electron microscopy revealed scattered, disconnected endocardial cells and a disorganized, loosely assembled myocardium (Figures 3h and 3i). Since endocardial defects preceded myocardial defects, and VE-cadherin was only detectable in endocardial but not in myocardial cells, the abnormal cardiac development most likely resulted from endocardial defects. A more detailed description of heart development in mutant embryos can be found in the Supplemental Data (see below).

Endothelial Cell–Cell Junctions

$VEC^{+/+}$ endothelial cells expressed molecules, present in tight junctions (JAM, ZO-1, occludin, cingulin), adherens-type junctions (VE-cadherin, β-catenin), or unrelated to junctional complexes (PECAM, N-cadherin) (Figure 1f; Figure 2; Figures 4a, 4c, and 4e; not shown). Mutant endothelial cells expressed comparable amounts of N-cadherin, JAM, ZO-1, cingulin, and PECAM (Figure 1f; Figure 2; Figures 4b and 4f; not shown) indicating that loss or truncation of VE-cadherin did not induce compensatory upregulation of these junctional molecules. In mutant cells, β-catenin was slightly reduced (Figure 1f) and not clustered at cell–cell contacts (Figures 4c and 4d) but associated with N-cadherin. Mutant endothelial cells were attached to each other by electron dense junctions (Figures 3c and 3d) that contained adhesive proteins found in tight junctions including JAM (Figure 2f) and ZO-1 and occludin (not shown). However, these intercellular contacts were less frequent, occurred over shorter distances, and were too weak to maintain an intact endothelial lining, resulting in numerous gaps between endothelial cells (Figures 3a and 3b).

Neutralization of VE-Cadherin in Cultured Embryos

Neutralization of VE-cadherin in cultured $VEC^{+/+}$ embryos using antibodies, that prevent clustering of VE-cadherin, confirmed the findings in mutant embryos. Injection of control or VE-cadherin antibodies in E7.5 embryos (e.g., at the onset of hemangioblast and endocard formation) did not prevent in situ differentiation of endothelial cells nor their assembly in vessels or in an intact endocardium (see Supplemental Data below). Instead, intracardial injection at E8.5 (e.g., when primitive vessels were already assembled) of VE-cadherin but not of control antibodies (or vehicle) caused abnormal remodeling of the vasculature with formation of blood lakes in the yolk sac, dilatation of head mesenchyme vessels, and detachment of the endocardium and other endothelial cells (Figures 2q and 2r). Thus, VE-cadherin is not required for initial endothelial assembly but is essential for normal vascular integrity and expansion/remodeling, even after a primitive vascular network is established.

Culturing of 3.5-day-old $VEC^{+/+}$ blastocysts into yolk sac cysts allowed differentiation of hemangioblasts into endothelial cells that subsequently assembled in primitive vessels. Endothelial cells also assembled in capillaries in $VEC^{-/-}$ yolk sacs as well as in $VEC^{+/+}$ yolk sac cysts treated with VE-cadherin antibodies. During prolonged culture, capillaries enlarged in $VEC^{+/+}$ yolk sacs but regressed in $VEC^{-/-}$ yolk sacs. Again, in this culture model, VE-cadherin function is not required for assembly of endothelial cells in vessels but is essential for their further expansion and maintenance. A more detailed description of vascular development in blastocyts can be found in the Supplemental Data (see below).

Role of VE-Cadherin in the Endothelial Survival Activity of VEGF

The mechanism via which VE-cadherin causes endothelial dysfunction was further studied. Differentiation of mutant endothelial cells was not impaired as suggested by the comparable expression of several endothelial

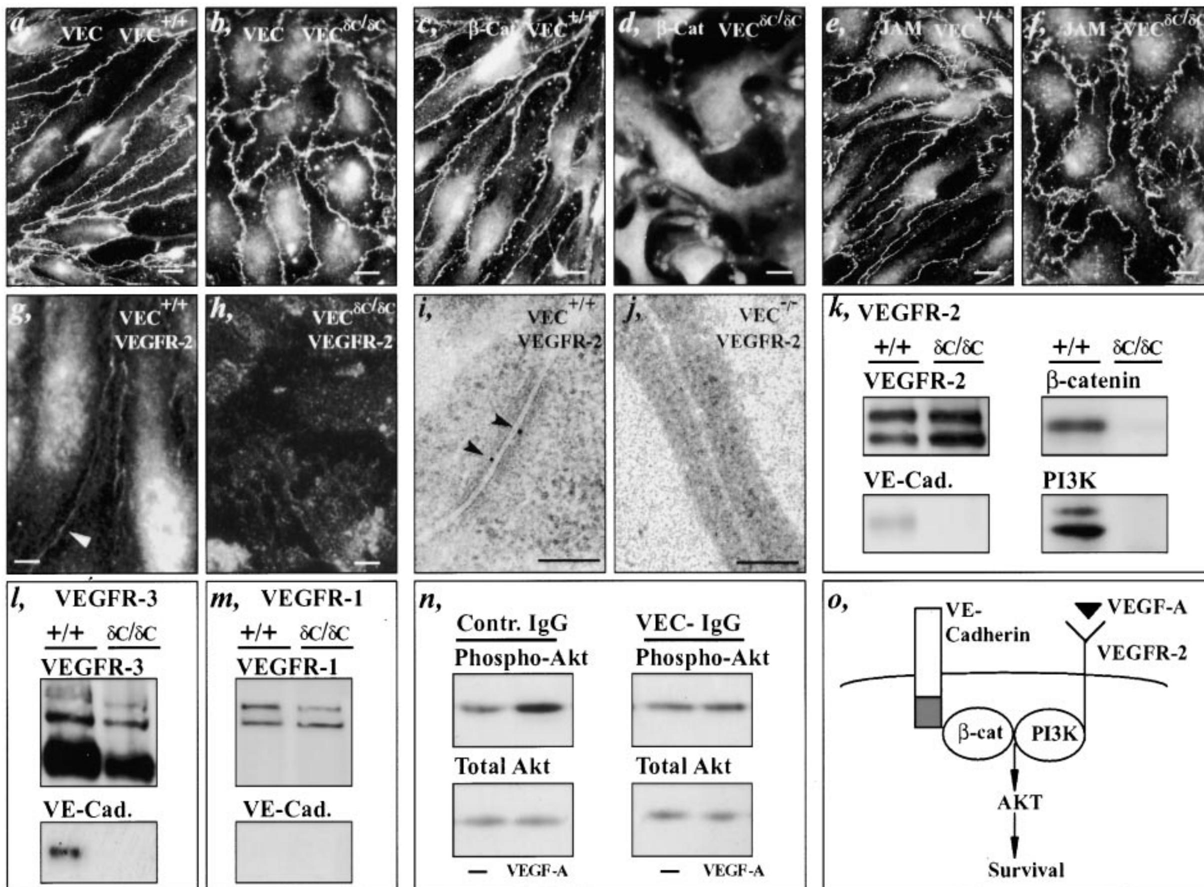

Figure 4. Expression of Junctional Proteins and Interaction of VE-Cadherin with VEGFR-2

(a–h) Immunofluorescent staining of cultured $VEC^{+/+}$ (a, c, e, and g) or $VEC^{\delta C/\delta C}$ (b, d, f, and h) endothelial cells for VE-cadherin (a and b), β-catenin (c and d), JAM (e and f) and VEGFR-2 (g and h), which were all clustered at junctions in $VEC^{+/+}$ cells. In contrast, β-catenin and VEGFR-2 were absent from endothelial junctions in $VEC^{\delta C/\delta C}$ cells. The residual white spots in (d) represent background.

(i and j) Immunogold labeling revealing VEGFR-2-labeled gold particles (arrowheads) at endothelial junctions from E9.0 $VEC^{+/+}$ (i) but not $VEC^{\delta C/\delta C}$ (j) embryos.

(k–m) Immunoprecipitation of cultured endothelial cells with antibodies to VEGFR-2 (k), VEGFR-3 (l), or VEGFR-1 (m) and subsequent Western blotting for the indicated proteins. VE-cad, VE-cadherin; PI3K, PI3-kinase. VEGFR-1 and VEGFR-2 exist as two different glycosylation forms; three VEGFR-3 polypeptides were detected: a 170 kDa precursor, a 190 kDa mature form, and a proteolytically cleaved 120 kDa form.

(n) Western blotting for phosphorylated (phospho-Akt) and total Akt in HUVEC cells without (−) or with VEGF-A in the presence of control or VE-cadherin IgG antibodies.

(o) Proposed model of the role of VE-cadherin in VEGF-A-mediated survival of endothelial cells. Via formation of a multicomponent junctional complex between VE-cadherin, β-catenin (β-cat), PI3-kinase (PI3K), and VEGFR-2, VE-cadherin promotes VEGF-A-mediated activation of Akt and endothelial survival.

Magnification bar is 1 μm in (i) and (j); 5 μm in (g) and (h); and 10 μm in (a)–(f).

receptors (see Supplemental Data below). Proliferation of endothelial cells in E9.0 yolk sacs was also similar (percent BrdU-positive cells: 20% ± 2% in $VEC^{+/+}$; 23% ± 4% in $VEC^{neo/neo}$; 22% ± 2% in $VEC^{-/-}$; and 25% ± 2% in $VEC^{\delta C/\delta C}$). However, there were fewer endothelial cells in E9.0 mutant than in $VEC^{+/+}$ yolk sacs (cells/mm sectioned yolk sac: 6 ± 2 in $VEC^{neo/neo}$; 7 ± 1 in $VEC^{-/-}$; 8 ± 1 in $VEC^{\delta C/\delta C}$; and 23 ± 2 in $VEC^{+/+}$; $p < 0.05$ versus $VEC^{+/+}$). The endothelial defects were not due to reduced expression of the angiogenic factors VEGF-A or VEGF-C (see Supplemental Data below). Therefore, vascular defects were most likely attributable to impaired endothelial survival.

When polyomavirus middle-T antigen-immortalized $VEC^{+/+}$ and $VEC^{\delta C/\delta C}$ endothelial cells were cultured in the absence of serum, $VEC^{\delta C/\delta C}$ endothelial cells exhibited increased apoptosis, measured as the number of TUNEL-positive cells (not shown) or as the amount of oligonucleosomes (Table 1). Notably, the endothelial survival factor VEGF-A rescued survival of $VEC^{+/+}$ but not of $VEC^{\delta C/\delta C}$ endothelial cells (Table 1), even though the latter cells expressed similar amounts of the VEGF-A receptor VEGFR-2 (Figure 4k). In contrast, basic fibroblast growth factor (bFGF) rescued endothelial survival regardless of the presence or absence of VE-cadherin (Table 1). The survival function of VE-cadherin was specific for VEGF-A, as other VEGF-A homologs including VEGF-C and VEGF-D (which bind VEGFR-3 and, less avidly, VEGFR-2) and placental growth factor (PLGF; which only binds VEGFR-1) had no effect on endothelial

Table 1. Effect of Growth Factors on Apoptosis, Bcl2, p53, and p21 in Immortalized Endothelial Cells

Growth Factors	Oligo-nucleosomes (per 10^5 cells)		Bcl2 (U/10^5 cells)		p53 (pg/10^5 cells)		p21 (pg/10^5 cells)	
	VEC$^{+/+}$	VEC$^{\delta C/\delta C}$	VEC$^{+/+}$	VEC$^{\delta C/\delta C}$	VEC$^{+/+}$	VEC$^{\delta C/\delta C}$	VEC$^{+/+}$	VEC$^{\delta C/\delta C}$
None	54 ± 3	160 ± 9	110 ± 10	33 ± 7	0.9 ± 0.1	1.6 ± 0.3	0.9 ± 0.1	1.9 ± 0.4
VEGF-A	20 ± 2*	190 ± 11	160 ± 8*	42 ± 6	0.4 ± 0.1*	1.1 ± 0.3	0.5 ± 0.1*	1.2 ± 0.3
VEGF-A + wortmannin	60 ± 5	180 ± 16	ND	ND	ND	ND	ND	ND
VEGF-A + LY294002	67 ± 6	180 ± 19	ND	ND	ND	ND	ND	ND
bFGF	11 ± 1*	47 ± 2*	155 ± 7*	90 ± 7*	0.4 ± 0.1*	0.6 ± 0.2*	0.6 ± 0.1*	0.6 ± 0.2*
PLGF	63 ± 4	190 ± 18	ND	ND	ND	ND	ND	ND
VEGF-C	64 ± 9	180 ± 13	ND	ND	ND	ND	ND	ND
VEGF-D	64 ± 9	180 ± 8	ND	ND	ND	ND	ND	ND

Values represent mean ± SD of at least six measurements. Growth factors were supplemented at 100 ng/ml; wortmannin and LY294002, at 100 nM. (*) $p < 0.05$ versus control without growth factors by unpaired t test. ND, not determined.

survival (Table 1), even though VEGFR-1 and VEGFR-3 were present (Figures 4l and 4m). The VE-cadherin/VEGF-A interaction was not restricted to immortalized murine endothelial cells, as treatment of freshly isolated human umbilical vein endothelial cells (HUVEC) or of the endothelial cell line 1G11 with VE-cadherin antibodies also abolished VEGF-A survival function. In addition, intravenous injection of VE-cadherin antibodies in adult wild-type mice, which leads to loss of vascular integrity and endothelial detachment in the heart (Corada et al., 1999), significantly increased the number of TUNEL-positive endothelial cells per mm^2: 1 ± 1 after injection of control antibodies versus 230 ± 20 after injection of VE-cadherin antibodies ($p < 0.05$ versus control antibodies) (Figures 3l and 3m).

VE-Cadherin/β-Catenin Signaling in PI3-Kinase/Akt-Mediated Survival

VEGF-A is a survival factor for endothelial cells via activation of VEGFR-2 and subsequent signaling of PI3-kinase (Gerber et al., 1998b). The PI3-kinase inhibitors wortmannin and LY294002 blocked VEGF-A-mediated survival of VEC$^{+/+}$ endothelial cells but did not affect apoptosis of VEC$^{\delta C/\delta C}$ endothelial cells when cultured in the absence of serum (Table 1). Immunofluorescent and immunogold labeling revealed that VEGFR-2 was present at intercellular junctions in VEC$^{+/+}$ cells but not in mutant endothelial cells (Figures 4g–4j). Coimmunoprecipitation experiments revealed an association between VE-cadherin, β-catenin, PI3-kinase and VEGFR-2 in VEC$^{+/+}$ but not in VEC$^{\delta C/\delta C}$ cells, notwithstanding comparable levels of VE-cadherin, VEGFR-2, PI3-kinase, and β-catenin (Figure 4k). Similar results were obtained when cell extracts were immmunoprecipitated with PI3-kinase, β-catenin, or VE-cadherin antibodies (not shown). VEGFR-3, but not VEGFR-1, also coimmunoprecipitated with VE-cadherin in VEC$^{+/+}$ but not in VEC$^{\delta C/\delta C}$ cells (Figures 4l and 4m).

In contrast to PLGF, VEGF-A induces PI3-kinase activity, known to result in phosphorylation of Akt (Gerber et al., 1998b). VEGF-A increased phospho-Akt levels ~4-fold in HUVEC cells treated with control but not with VE-cadherin antibodies, despite similar total Akt kinase levels (arbitrary densitometric units: 720 control and 2100 after VEGF-A for control antibodies versus 660 control and 740 after VEGF-A for VE-cadherin antibodies; Figure 4n). Phospho-Akt levels were also increased by VEGF-A in VEC$^{+/+}$ but not in VEC$^{\delta C/\delta C}$ cells (800 control and 1100 after VEGF-A in VEC$^{+/+}$ cells versus 450 control and 300 after VEGF-A in VEC$^{\delta C/\delta C}$ cells; the constitutive activation of Akt by polyomavirus middle T-antigen used for cell immortalization [Meili et al., 1998] most likely impaired Akt induction in response to VEGF-A). Total Akt was immunolocalized in endothelial cells in VEC$^{+/+}$ and mutant embryos but, due to lack of suitable antibodies, phospho-Akt could not be immunolocalized in vivo (see Supplemental Data below). Despite the presence of VEGFR-3 in the VE-cadherin complex, VEGF-C did not affect endothelial apoptosis (Table 1) nor Akt phosphorylation (densitometry levels: 800 control and 600 after VEGF-C in VEC$^{+/+}$ versus 610 control and 550 after VEGF-C in VEC$^{\delta C/\delta C}$). Thus, a multicomponent complex comprising VE-cadherin, β-catenin, VEGFR-2, and PI3-kinase appears to be required for the endothelial survival function of VEGF-A through activation of Akt. Disruption of this complex by truncation of VE-cadherin (which abolishes association with β-catenin) or by VE-cadherin antibodies (which prevents clustering of VE-cadherin) renders endothelial cells refractory to the VEGF-A survival signal (Figure 4o).

Role of p53/p21 and Bcl2 in VE-Cadherin-Mediated Endothelial Survival

Endothelial apoptosis has been associated with downregulation of Bcl2 and upregulation of the proapoptotic mediator p53 and its target p21 (Stromblad et al., 1996; Gerber et al., 1998a). VEGF-A increased the levels of Bcl2 and reduced those of p53 and p21 in VEC$^{+/+}$ but not in VEC$^{\delta C/\delta C}$ cells, when cultured in the absence of serum (Table 1). In contrast, bFGF increased the levels of Bcl2 and reduced those of p53/p21 in both genotypes (Table 1). Treatment of serum-starved HUVEC cells with VE-cadherin but not with control antibodies also reduced Bcl2 and increased p53/p21 levels and abrogated the survival effect of VEGF-A but not of bFGF (see Supplemental Data below). p53 and p21 immunoreactivity were detected in mutant but not in VEC$^{+/+}$ endothelial cells in E9.0 yolk sacs (Figures 3n and 3o) and in adult myocardial endothelial cells after intravenous injection of VE-cadherin but not of control antibodies (Figures 3p and 3q). In addition, *p21* mRNA transcripts, determined by quantitative real-time RT-PCR, were higher in mutant than in VEC$^{+/+}$ yolk sacs (copies *p21* per 1000 copies *hprt*: 340 ± 30 in VEC$^{-/-}$ versus 180 ± 19 in VEC$^{+/+}$ at

E8.75; 200 ± 40 in VEC$^{-/-}$ versus 100 ± 9 in VEC$^{+/+}$ at E9.5; $p < 0.05$). No differences were observed in *p53* mRNA transcript levels (copies *p53* per 1000 copies *hprt*: 2100 ± 200 in VEC$^{+/+}$ versus 2300 ± 240 in VEC$^{-/-}$ at E8.75; p = NS), suggesting that p53 levels may be regulated posttranscriptionally. Mice deficient in p53 or p21 contained fewer TUNEL-positive endothelial cells than wild type mice after intravenous injection of VE-cadherin antibodies (TUNEL-positive endothelial cells/mm^2 were 230 ± 20 in wild type, 160 ± 15 in p53$^{-/-}$, $p < 0.05$ versus wild type; and 95 ± 20 in p21$^{-/-}$, $p < 0.05$ versus wild type).

Hematopoiesis

Loss or truncation of VE-cadherin did not significantly impair hematopoiesis in E8.5 yolk sacs, as comparable numbers of nucleated blood cells were present in blood islands in vivo, and a similar number of hematopoietic progenitors was present in yolk sacs: the number of erythroid (BFU-E), myeloid (CFU-GM), or mixed (CFU-mix) colonies per 10^4 yolk sac cells was 34 ± 8, 18 ± 7, and 37 ± 10 in VEC$^{+/+}$ yolk sacs and 33 ± 7, 23 ± 7, and 38 ± 10 in VEC$^{-/-}$ yolk sacs (p = NS).

Supplemental Data

Due to space restrictions, supplemental data (http://www.cell.com/cgi/content/full/98/2/147/DC1) provide more detailed information on the description of the cardiovascular development in mutant embryos, on the expression of endothelial markers and angiogenic candidates, on vascular formation in cultured embryos and blastocysts, on the immunolocalization of Akt, and on Bcl2 and p53/p21 levels in mutant endothelial cells.

Discussion

Although several adhesive molecules attach vascular cells to each other, VE-cadherin plays a unique, essential role in this process, since loss or truncation of VE-cadherin prevents normal vascular development. This is unexpected, as N-cadherin also has strong adhesive properties (Radice et al., 1997) and is associated with the same set of proteins in mutant endothelial cells. The present study confirms the well-known role of VE-cadherin in mediating interendothelial adhesion but in addition unveils a novel role for this molecule in controlling VEGF-A-mediated endothelial survival, via a pathway involving the cytoplasmic domain of VE-cadherin, β-catenin, PI3-kinase, and VEGFR-2.

Loss or truncation of VE-cadherin was expected to abort vascular development in its earliest stage in vivo, as VE-cadherin-deficient endothelial cells failed to organize in vessel-like patterns, at least in an artificial embryoid body system in vitro (Vittet et al., 1997). However, our data demonstrate that neither VE-cadherin nor the stabilization of the adherens-type junctions, which depends on the interaction of the VE-cadherin tail with the cytoskeleton, is required for initial assembly of endothelial cells in vivo. This may relate to the minimal hemodynamic stress before onset of the circulation and/or to the presence of other adhesive molecules at interendothelial contacts.

Although enlargement of certain vessels in mutant embryos may be due to insufficient endothelial adhesion, most vascular defects in mutant embryos appeared attributable to impaired intracellular signaling. This is suggested by appearance of vascular defects in mutant embryos before onset of flow (E8.5) or in the absence of vitello-embryonic circulation (>E8.75), by abnormal expansion and remodeling into a mature vascular network, by impaired sprouting, by vessel regression, by reduced endothelial survival, and, most notably, by the similar phenotype of embryos completely lacking or expressing a truncated VE-cadherin.

Loss or truncation of VE-cadherin did not impair endothelial proliferation or differentiation, but increased endothelial apoptosis due to an inability of VE-cadherin deficient cells to respond to the survival activity of VEGF-A. The latter is known to mediate endothelial survival via binding to VEGFR-2, thereby activating PI3-kinase and Akt (Nunez and del Peso, 1998) and via increasing the levels of the antiapoptotic mediator Bcl2 (Gerber et al., 1998a, 1998b). VE-cadherin appears to be required for these VEGF-A-dependent survival signals through formation of a VE-cadherin/β-catenin/PI3-kinase/VEGFR-2 complex. Interestingly, one of the first signs of endothelial apoptosis is rapid cleavage of VE-cadherin and β-catenin (Brancolini et al., 1997; Herren et al., 1998), which might represent an amplification mechanism to the death response.

Previous studies have implicated nuclear signaling of β-catenin in apoptosis but its role remains controversial. Indeed, reduced stabilization of free cytosolic β-catenin, due to impaired interaction with mutated presenilin, appeared to potentiate neuronal apoptosis (Zhang et al., 1998) whereas increased *Drosophila* β-catenin, complexed with Tcf, activated apoptosis (Ahmed et al., 1998). In the present study, β-catenin levels were slightly reduced in VEC$^{-/-}$ and VEC$^{\delta C/\delta C}$ endothelial cells, and the ratio of β-catenin bound to VE-cadherin relative to that present in the cytoplasm or the nucleus did not change in antibody-treated VEC$^{+/+}$ cells (not shown), rendering it unlikely that susceptibility to apoptosis was due to increased levels of free β-catenin.

Refractoriness of endothelial cells to VEGF-A survival signals may largely explain the mutant phenotype. Once endothelial cells are assembled and organized in primitive vessels, subsequent remodeling and pruning of the primitive vascular labyrinth may require endothelial apoptosis. The present study demonstrates that VE-cadherin is not essential during initial vessel assembly but that it regulates endothelial apoptosis during the second phase of angiogenesis. Interestingly, the role of VE-cadherin is specific for VEGF-A and not for bFGF, a pleiotrophic factor that also affects nonendothelial cells, thereby rendering the VEGF-A/VE-cadherin response highly restricted to endothelial cells. The expression of VEGFR-2 and VE-cadherin in all endothelial cells and of VEGF-A in the surrounding mesenchyme in the E8.5–E9.5 embryo (Breier et al., 1995, 1996) underscores the role of VE-cadherin in controlling the VEGF-A/VEGFR-2 survival function during vascular development.

The role of VEGFR-3 clustered to VE-cadherin in the junction and its relevance for the present phenotype is less obvious. Indeed, although VEGFR-3 codistributed with VE-cadherin in junctions, VEGF-C did not affect

endothelial survival nor Akt phosphorylation. Furthermore, VEGFR-3 expression is restricted to the venous endothelium and absent in the dorsal aorta, the endocard, and yolk sac endothelial cells (Kukk et al., 1996), which were all affected in mutant embryos. Perhaps, junctional VEGFR-3 requires other signals for survival or could be involved in other processes.

Endothelial apoptosis is increasingly recognized as a possible mechanism of action of angiogenesis inhibitors such as $\alpha_v\beta_3$ antagonists, thrombospondin, angiostatin, endostatin, etc. (Stromblad et al., 1996). Nevertheless, the intracellular signals mediating endothelial apoptosis remain incompletely understood. The upregulation of p53/p21 in apoptosis-prone VE-cadherin-deficient endothelial cells suggests a possible causal role of these signals in endothelial apoptosis. However, since endothelial apoptosis in response to immunoneutralizing VE-cadherin antibodies was only partially reduced in $p53^{-/-}$ or $p21^{-/-}$ mice, other proapoptotic pathways may be implicated as well.

An intriguing issue is whether VE-cadherin also exerts a survival or maintenance function for adult quiescent endothelium or during pathological angiogenesis. The constitutive life-long expression of VE-cadherin, in addition to our present data that VE-cadherin antibodies induce endothelial apoptosis in adult mice, would indeed support such a role. In conclusion, loss of VE-cadherin or lack of binding of β-catenin via truncation of its cytoplasmic tail prevents vascular development due to an impaired response of endothelial cells to VEGF-A survival signals. The data implicate a novel role for VE-cadherin/β-catenin in intracellular signaling by clustering proteins involved in VEGF-A survival signaling.

Experimental Procedures

Generation of Transgenic Mice

pPNT.VEC^{null} contained a 3.1 kb BglII fragment, a floxed *neo* cassette, and a 5.5 kb XbaI/EcoRV fragment (Figure 1a). pPNT.$VEC^{\delta C}$ contained a 5.8 kb XbaI/EcoRI fragment, a floxed *neo* cassette, and a 2.1 kb EcoRI fragment. The oligonucleotide T**TAG**TCTAGATGGGCC (stop codon, bold; XbaI site, underlined) was inserted at nucleotide 2141 of the *VE-cadherin* cDNA (Figure 1a). The following primers were used for PCR genotyping: VEC1, 5′-GTC CAA CGT GAA CCG CCA GAA TGC TAA-3′; VEC2, 5′-GAC ATC TCT GGC ACA GAT GCG TTG AAT-3′; PGKpA, 5′-AAT GTG TCA GTT TCA TAG CC-3′; VEC3, 5′-ACC GTG GGT GTG TGC AAG-3′; VEC4, 5′-AGT GAA TTC TTT CTT CAC GTC GAT CAT GGT G-3′; VEC5, 5′-CTG TTG CTC TGA CTT GCA GTG ATT TTG CTG AT-3′; VEC6, 5′-GGC CAT CTC CCT GGG CCC ATC TAG ACT AA-3′; VEC7, 5′-GCC TGG ACT GCA CGG AGG GCC CAG GGA G-3′; VEC8, 5′-TCT CAC TCT TCC AGT TTT TGG AGT GGC-3′ (Figures 1a and 1b). $VEC^{\delta C/\delta C}$ mice were generated by crossbreeding $VEC^{+/neo}$ mice with *pgk:cre* mice (Lallemand et al., 1998). All methods for culture, selection, identification of ES cells, and generation of transgenic mice have been described (Carmeliet et al., 1996a; Rosen et al., 1997). Western blotting and quantitative real-time RT-PCR was performed as described (Rosen et al., 1997; Carmeliet et al., 1999). Primers for RT-PCR were for p21, 5′-GTT CCG CAC AGG AGC AAA G′3, 5′-ACG GCG CAA CTG CTC ACT-3′; and for p53, 5′-TTC TTC CCT CAA TAA GCT ATT CTG-3′, 5′-CCG TCA TGT GCT GTG ACT TCT T-3′; probes were for p21, 5′-CGT TGT CTC TTC GGT CCC GTG GA-3′, and for p53, 5′-ACA ACT GCA CAG GGC ACG TCT TCG-3′.

Histology, Immunostaining, and Electron Microscopy

All methods for histology, immunostaining, and transmission and scanning electron microscopy have been described (Carmeliet et al., 1996b; Rosen et al., 1997; Esser et al., 1998; Hogers et al., 1999). For immunostaining of VEGFR-2, cultured cells were fixed in −20°C methanol (3 min), permeabilized with saponin (15 min), and incubated with 0.5% BSA–0.1% saponin in PBS before incubation with antibodies. The following antisera were used: rat anti-CD31/PECAM (clone MEC7.46), rat anti-VE-cadherin (clone 19E6), rat anti-JAM (clone BV11), mouse anti-desmin (ICN Pharmaceuticals #10519, Costa Mesa, CA), rabbit anti-laminin (Sigma), mouse anti-β-catenin (clone 14) and anti-ZO-1 (both from Transduction Laboratories, Lexington, KY), rabbit anti-N-cadherin and rabbit anti-pancadherin (both gift from Dr. Geiger), rabbit anti-mouse Akt (NEB, Beverly, MA), rabbit anti-mouse p53 (Novo Castro #CMTp, Newcastle, UK), rabbit anti-p21/WAF (Oncogene Research Products #PC55, Cambridge, MA). Mouse monoclonal antibodies were biotinylated.

Culture of Embryos and Endothelial Cells

Methods for culturing E7.5 and E8.5 embryos have been described (Rosen et al., 1997; Van Maele-Fabry et al., 1997), except that a culture medium consisting of 50% DMEM and 50% rat serum was used for E7.5 embryos. Fifty nanoliters (E7.5) or 100 nl (E8.5) Hanks′ balanced salt solution (HBSS; vehicle) containing 100 μg/ml antibodies was injected in the region immediately anterior to the right cardiogenic mesoderm (E7.5) or intracardially (E8.5) (Rosen et al., 1997). Noninjected E7.5 embryos were also cultured in medium containing 100 μg/ml control or VE-cadherin antibodies. After 16 (E7.5) or 8 (E8.5) hr of culture, embryos were processed for histology. Preimplantation blastocysts (3.5 days old) were cultured under conditions supporting their development into yolk sac cysts as described (McClanahan et al., 1993). Differentiation of ES cells to endothelial cells and enrichment of endothelial cells were performed as described (Vittet et al., 1996). Immortalized endothelial cell lines were generated by transduction of endothelial cells, obtained by flow-cytometric enrichment of differentiated ES cells, with a retrovirus expressing the Polyoma middle-T oncogene (Bussolino et al., 1991). Alternatively, E9.0 embryos were enzymatically dispersed using collagenase A (1.5 mg/ml; Boehringer Mannheim, Germany) and subsequently infected with the Polyoma middle-T retrovirus. Endothelial cells were routinely cultured on 0.1% gelatin-coated dishes in DMEM with 20% fetal calf serum (Life Technologies, Paisley, UK), supplemented with endothelial cell growth supplement (50 μg/ml; Life Technologies) and heparin (100 μg/ml; Sigma) (Esser et al., 1998).

Apoptosis and Proliferation

For apoptosis, endothelial cells were seeded at 2.4×10^5 cells for 24 hr in 24-well dishes in 0.5 ml of serum-free MCDB 131 medium (Life Technologies, Paisley, UK) supplemented with 1% bovine serum albumin (BSA) and ITS (Life Technologies). The culture was continued for 96 hr either in the absence or in the presence of VEGF-A or PLGF (R&D Systems, MN; 30–100 ng/ml), bFGF (R&D; 50 ng/ml), or VEGF-C (100 ng/ml; gift of K. Alitalo). Antibody to VE-cadherin (clone BV13) or control antibody (both at 50 μg/ml) were added 24 hr after seeding, and daily thereafter. Apoptosis was quantified by measuring cytoplasmic histone–associated DNA fragments (mono- and oligonucleosomes) or TUNEL, and Bcl2 (U/10^5 cells), p21 (U/10^5 cells), and p53 (pg/10^5 cells) were quantified as described (Carmeliet et al., 1998). Apoptosis in embryos was visualized by TUNEL or annexin V immunostaining after intracardiac injection of 100 nl AnX-biotin (Apoptest-biotin; Nexins Research B. V., Hoeven, The Netherlands). The number of proliferating yolk sac endothelial cells was expressed as percent of total (Carmeliet et al., 1996a). Antibody injections in adult mice was done as described (Corada et al., 1999) and hearts were analyzed for TUNEL, p53, and p21 immunostaining after 16 hr.

Measurement of β-Catenin, PI3-Kinase, and Akt Levels

For coimmunoprecipitation, endothelial cells (2.4×10^6 in 25 cm^2 flasks) were cultured for 48 hr in MCDB 131 medium containing 1% BSA, and VEGF-A (80 ng/ml) was added during the last 25 min and pervanadate (vanadate 100 μM; hydrogen peroxide 200 μM) during the last 7 min. Extraction, immunoprecipitation, and Western blotting for N-cadherin, β-catenin, VEGFR-2, VEGFR-1, VEGFR-3 (Santa

Cruz Biotechnology, Santa Cruz, CA), PI3-K (mouse antibody; Transduction laboratories), and VE-cadherin were performed as described (Esser et al., 1998). To determine Akt phosphorylation (PhospoPlus Akt-Ser473 kit, NEB, Westburg, Netherlands), endothelial cells (10^6 cells in 35 cm Petri dishes) were cultured and stimulated as described above, except that cells were cultured for 5 days before stimulation (30–100 ng/ml VEGF-A or VEGF-C) and that pervanadate was omitted.

Acknowledgments

The authors thank Dr. K. Alitalo for VEGF-C, Dr. S. Soddu for $p53^{-/-}$ mice, Dr. P. G. Pelicci for $p21^{-/-}$ mice, Dr. S. Citi for cingulin antibodies, Dr. P. Lonai for pgk:Cre mice, Dr. D. Hicklin and P. Bohlen (Imclone System, Inc.) for support. This work was supported by Associazione Italiana per la Ricerca sul Cancro, the European Community (Biomed BMH4-CT98–3380), Actie Levenslijn (#7.0019.98), and the Italian National Research Council (CNR 97.01299.PF49). M. G. L. is a recipient of a NATO travel grant.

Received March 15, 1999; revised June 8, 1999.

References

Ahmed, Y., Hayashi, S., Levine, A., and Wieschaus, E. (1998). Regulation of armadillo by a *Drosophila* APC inhibits neuronal apoptosis during retinal development. Cell *93*, 1171–1182.

Balsamo, J., Leung, T., Ernst, H., Zanin, M.K., Hoffman, S., and Lilien, J. (1996). Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. J. Cell Biol. *134*, 801–813.

Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature *382*, 638–642.

Brancolini, C., Lazarevic, D., Rodriguez, J., and Schneider, C. (1997). Dismantling cell–cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. J. Cell Biol. *139*, 759–771.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnurch, 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*, 630–641.

Breier, G., Clauss, M., and Risau, W. (1995). Coordinate expression of vascular endothelial growth factor receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. Dev. Dyn. *204*, 228–239.

Bussolino, F., De Rossi, M., Sica, A., Colotta, F., Wang, J.M., Bocchietto, E., Padura, I.M., Bosia, A., DeJana, E., and Mantovani, A. (1991). Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. J. Immunol. *147*, 2122–2129.

Carmeliet, P., Ng, Y.S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V.V., Stalmans, I., Mattot, V., et al. (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking VEGF164 and VEGF188. Nat. Med. *5*, 495–502.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation, and tumor angiogenesis. Nature *394*, 485–490.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Kendraprasad, H., Eberhardt, C., et al. (1996a). Abnormal blood vessel development and lethality in embryos lacking a single vascular endothelial growth factor 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., et al. (1996b). Role of tissue factor in embryonic blood vessel development. Nature *383*, 73–75.

Caveda, L., Martin-Padura, I., Navarro, P., Breviario, F., Corada, M., Gulino, D., Lampugnani, M.G., and Dejana, E. (1996). Inhibition of cultured cell growth by vascular endothelial cadherin (cadherin-5/VE-cadherin). J. Clin. Invest. *98*, 886–893.

Corada, M., Moriotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., Lampugnani, M.G., Martin-Padura, I., Stoppacciaro, A., Ruco, L., et al. (1999). VE-cadherin is an important determinant of microvascular permeability in vivo. Proc. Natl. Acad. Sci. USA, in press.

Dejana, E. (1996). Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. J. Clin. Invest. *98*, 1949–1953.

Esser, S., Lampugnani, M.G., Corada, M., Dejana, E., and Risau, W. (1998). Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J. Cell Sci. *111*, 1853–1865.

Fagotto, F., Funayama, N., Gluck, U., and Gumbiner, B.M. (1996). Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in Xenopus. J. Cell Biol. *132*, 1105–1114.

Gerber, H.P., Dixit, V., and Ferrara, N. (1998a). Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J. Biol. Chem. *273*, 13313–13316.

Gerber, H.P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B.A., Dixit, V., and Ferrara, N. (1998b). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J. Biol. Chem. *273*, 30336–30343.

Gumbiner, B.M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell *84*, 345–357.

Herren, B., Levkau, B., Raines, E.W., and Ross, R. (1998). Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: evidence for a role for caspases and metalloproteinases. Mol. Biol. Cell *9*, 1589–1601.

Hogers, B., De Ruiter, M.C., Gittenberger-de Groot, A.C., and Poelmann, R.E. (1999). Extraembryonic venous obstructions lead to cardiovascular malformations and can be embryolethal. Cardiovasc. Res. *41*, 87–99.

Hoschuetzky, H., Aberle, H., and Kemler, R. (1994). Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J. Cell Biol. *127*, 1375–1380.

Hynes, R.O. (1992). Specificity of cell adhesion in development: the cadherin superfamily. Curr. Opin. Genet. Dev. *2*, 621–624.

Kemler, R. (1992). Classical cadherins. Semin. Cell Biol. *3*, 149–155.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., and Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development *122*, 3829–3837.

Lallemand, Y., Luria, V., Haffner-Krausz, R., and Lonai, P. (1998). Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase. Transgenic Res. *7*, 105–112.

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.

McClanahan, T., Dalrymple, S., Barkett, M., and Lee, F. (1993). Hematopoietic growth factor receptor genes as markers of lineage commitment during in vitro development of hematopoietic lineages. Blood *81*, 2903–2915.

Meili, R., Cron, P., Hemmings, B.A., and Ballmer-Hofer, K. (1998). Protein kinase B/Akt is activated by polyomavirus middle-T antigen via a phosphatidylinositol 3-kinase-dependent mechanism. Oncogene *16*, 903–907.

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.

Nieset, J.E., Redfield, A.R., Jin, F., Knudsen, K.A., Johnson, K.R.,

and Wheelock, M.J. (1997). Characterization of the interactions of alpha-catenin with alpha-actinin and beta-catenin/plakoglobin. J. Cell Sci. *110*, 1013–1022.

Nunez, G., and del Peso, L. (1998). Linking extracellular survival signals and the apoptotic machinery. Curr. Opin. Neurobiol. *8*, 613–618.

Nusse, R. (1997). A versatile transcriptional effector of wingless signaling. Cell *89*, 321–323.

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.

Rosen, E., Chan, J.Y., Esohe, I., Clotman, F., Vlasuk, G., Albrecht, S., Lissens, A., Luther, T., Jalbert, L., Schoonjans, L., et al. (1997). Factor VII deficient mice develop normally but suffer fatal perinatal bleeding. Nature *390*, 290–294.

Salomon, D., Ayalon, O., Patel-King, R., Hynes, R.O., and Geiger, B. (1992). Extrajunctional distribution of N-cadherin in cultured human endothelial cells. J. Cell Sci. *102*, 7–17.

Stromblad, S., Becker, J.C., Yebra, M., Brooks, P.C., and Cheresh, D.A. (1996). Suppression of p53 activity and $p21^{WAF1/CIP1}$ expression by vascular cell integrin $\alpha V\beta 3$ during angiogenesis. J. Clin. Invest. *98*, 426–433.

Van Maele-Fabry, G., Clotman, F., Gofflot, F., Bosschaert, J., and Picard, J.J. (1997). Postimplantation mouse embryos cultured in vitro. Assessment with whole-mount immunostaining and in situ hybridization. Int. J. Dev. Biol. *41*, 365–374.

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.

Zhang, Z., Hartmann, H., Do, V.M., Abramowski, D., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., van de Wetering, M., Clevers, H., Saftig, P., et al. (1998). Destabilization of beta-catenin by mutations in presenilin-1 potentiates neuronal apoptosis. Nature *395*, 698–702.