Deciphering the functional role of endothelial junctions by using *in vivo* models

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Endothelial cell-to-cell junctions are vital for the formation and integrity of blood vessels. The main adhesive junctional complexes in endothelial cells, adherens junctions and tight junctions, are formed by transmembrane adhesive proteins that are linked to intracellular signalling partners and cytoskeletal-binding proteins. Gene inactivation and blocking antibodies in mouse models have revealed some of the functions of the individual junctional components *in vivo*, and are increasing our understanding of the functional role of endothelial cell junctions in angiogenesis and vascular homeostasis. Adherens-junction organization is required for correct vascular morphogenesis during embryo development. By contrast, the data available suggest that tight-junction proteins are not essential for vascular development but are necessary for endothelial barrier function.

Keywords: adherens junctions; endothelial cells; tight junctions; VE-cadherin

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Introduction

The organization of adhesive structures between endothelial cells occurs during the early stages of vascular development and is crucial for the formation of the vascular network. Intercellular junctions not only provide attachment sites, but also transfer intracellular signals that promote vascular stabilization and contribute to correct vascular morphogenesis. Endothelial cells have at least two types of specialized junction: adherens junctions (AJs) and tight junctions (TJs; Dejana, 2004; Hartsock & Nelson, 2007; Wallez & Huber, 2007; Vestweber, 2008). Adherens junctions are important for vascular development and remodelling, whereas an important function of TJs is the control of endothelial barrier properties. Several transmembrane and intracellular proteins form the architecture of AJs and TJs (Fig 1). One of the main adhesive components

of endothelial AJs is vascular endothelial (VE)-cadherin, which mediates homophilic adhesion between adjacent endothelial cells (Dejana, 2004; Hartsock & Nelson, 2007; Wallez & Huber, 2007; Vestweber, 2008). Through its cytoplasmic domain, VE-cadherin associates directly with β -catenin, plakoglobin and p120^{cas}, and indirectly with α -catenin. Several receptor and cytoplasmic protein tyrosine phosphatases (PTPs) localize at AJs and can dephosphorylate components of the cadherin–catenin complex (Wallez & Huber, 2007; Vestweber, 2008). At TJs, adhesion is mediated by different proteins including claudins, occludin and junctional adhesion molecules (JAMs), which interact directly or indirectly with cytoplasmic partners such as the zonula occludens (ZO) proteins and cingulin (Fig 1B). The molecular organization of AJs and TJs in endothelial cells has already been described in detail (Bazzoni & Dejana, 2004).

The function of these structures in the control of permeability and vessel growth has been studied both *in vitro*, using cultured endothelial cells, and *in vivo*, using blocking antibodies or genetically modified mice (Table 1). In this review, we summarize the *in vivo* experiments in which components of endothelial cell junctions have been blocked or deleted.

Cadherins

VE-cadherin belongs to the group of classic cadherins and is the only member that is restricted to the endothelium (Lampugnani *et al*, 1992). Deletion of the VE-cadherin gene in mice resulted in embryonic lethality at mid-gestation with severe defects in vascular remodelling (Carmeliet *et al*, 1999; Gory-Faure *et al*, 1999). During the early phases of vascular development, the absence of VE-cadherin did not prevent angioblast differentiation or the formation of the vascular primitive plexus, but the subsequent vascular remodelling was severely affected. VE-cadherin-null endothelial cells gradually disconnected from each other and detached from the basement membrane, leading to vessel collapse, regression and large haemorrhages. Several vessels presented an enlarged and/or irregular lumen, indicating that VE-cadherin has an important role in the control of endothelial cell polarization and proliferation.

Studies performed on isolated allantoises (Crosby *et al*, 2005) showed that VE-cadherin is crucial for vascular stabilization. Inactivation of the VE-cadherin gene or a block of its adhesive function induced rapid vascular disassembly with the formation

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A Adherens junction complex

B Tight junction complex

Fig 1 | Schematic representations of endothelial junction complexes. (A) Adherens junction (AJ) complex. (B) Tight junction (TJ) complex. Vascular endothelial (VE)-cadherin, and in some cases neuronal (N)-cadherin, promotes cell–cell adhesion, and binds to different intracellular partners that contribute to intracellular signalling and re-shaping of the actin cytoskeleton. α -Catenin binds to either β -catenin or actin microfilaments and promotes actin bundling. VE-protein tyrosine phosphatase (VE-PTP) associates specifically with VE-cadherin. Claudins promote cell–cell adhesion at TJs with the cooperation of other proteins such as occludin and junctional adhesion molecules (JAMs). Zonula occludens (ZO)1, ZO2 and cingulin contribute to TJ interaction with the actin cytoskeleton. Nectins and their intracellular partners, such as afadin, contribute to AJ and TJ organization. Details of the roles of each molecule are reported in the text. CASK, calcium/calmodulin-dependent serine protein kinase; ESAM, endothelial-cell-selective adhesion molecule; MAGI, membrane-associated guanylate kinase inverted; MUPP1, multi-PDZ protein 1; PAR3/6, partitioning-defective proteins; RPTP μ , receptor-like protein tyrosine phosphatase μ ; SHP2, protein tyrosine phosphatase, non-receptor type 2; ZONAB, Y-box transcription factor.

of endothelial cell clusters. Interestingly, blocking VE growth factor (VEGF) activity resulted in similar morphological changes in the allantoises, supporting the idea of a link between VE-cadherin and VEGF signalling (Lampugnani *et al*, 2003; Weis *et al*, 2004; Lampugnani *et al*, 2006).

Neuronal (N)-cadherin is also expressed at high levels in endothelial cells (Liaw *et al*, 1990; Salomon *et al*, 1992). Whereas VE-cadherin is mainly involved in mediating adhesion between adjacent endothelial cells and is located at AJs, N-cadherin has a mostly dispersed distribution on cell membranes. In the presence of VE-cadherin, N-cadherin is excluded from junctions; however, in its absence, N-cadherin expression is increased and concentrated at AJs (Navarro *et al*, 1998). This exclusive distribution pattern of N-cadherin and VE-cadherin is obvious in stabilized long-confluent cells and, *in vivo*, in adult mice. However, at early stages of confluency, the two cadherins can both localize to AJs (Luo & Radice, 2005).

Endothelial-specific inactivation of N-cadherin leads to a vascular phenotype similar to that of VE-cadherin knockout (Luo & Radice, 2005). These data were unexpected because the lethality of N-cadherin-null embryos was attributed essentially to problems of heart development (Radice *et al*, 1997; Luo *et al*, 2001). *In vitro* data obtained using RNA interference (RNAi) indicate that N-cadherin acts upstream from VE-cadherin and modulates its expression by a post-transcriptional mechanism (Luo & Radice, 2005). However,

others, using an *in vitro* model of endothelial development within stem-cell-derived embryoid bodies, have observed that, in contrast to VE-cadherin-null cells, N-cadherin-null endothelial cells are still able to sprout and form vascular structures (Vittet *et al*, 1997).

N-cadherin might also be important for the interaction between endothelial cells and pericytes. In the developing chick brain, blocking antibodies to N-cadherin disrupted endothelial–pericyte interaction, and caused vascular abnormalities and haemorrhages (Gerhardt *et al*, 2000; Gerhardt & Betsholtz, 2003). Furthermore, Tillet and co-workers showed that in embryonic-stem-cell-derived angiogenesis, a lack of N-cadherin prevents pericytes from covering endothelial outgrowths (Tillet *et al*, 2005). These results point to a complex role for N-cadherin in mediating recognition and signalling between endothelial and perivascular cells.

Catenins

Cadherins associate directly with several cytoplasmic proteins. β -Catenin and plakoglobin are two closely related members of the armadillo family of proteins that bind to the distal region of the cytoplasmic cadherin tail (Fig 1A). Cadherin-bound β -catenin or plakoglobin can bind α -catenin and form a ternary complex (Aberle *et al*, 1994; Yamada *et al*, 2005). α -Catenin can also bind to actin filaments, but not when bound to β -catenin (Yamada *et al*, 2005). It has been proposed that the cadherin–catenin complex indirectly influences the actin cytoskeleton by increasing the α -catenin local

Table 1 | Knockout mouse models of junctional components

Junctions and proteins	Phenotypes	References
<i>Adherens junctions</i>		
VE-cadherin	Severe defects in vascular remodelling; vessels collapse and regress, and large haemorrhages occur; endothelial cell apoptosis	Carmeliet <i>et al</i> , 1999 Gory-Faure <i>et al</i> , 1999
N-cadherin-endothelial restricted	Severe vascular defects; reduced endothelial cell proliferation, reduced cell motility and weak vessel wall	Luo & Radice, 2005
β -Catenin-endothelial restricted	Defective vascular patterning and integrity; altered junctional organization	Cattellino <i>et al</i> , 2003
Plakoglobin	No vascular phenotype	Ruiz <i>et al</i> , 1996 Bierkamp <i>et al</i> , 1996
Desmoplakin	Altered microvascular development	Gallicano <i>et al</i> , 2001
<i>Adherens-junctions-associated protein tyrosine phosphatases</i>		
VE-PTP	Severe defects in vascular remodelling; hyperfusion of yolk-sac vessels	Baumer <i>et al</i> , 2006 Dominguez <i>et al</i> , 2007
CD148 (DEP1/PTP η)	Defects during vasculogenesis; severe defects in vascular remodelling; hyperfusion of yolk-sac vessels	Takahashi <i>et al</i> , 2003
RPTP μ	No effects on vascular morphology, but impaired flow-induced dilation in mesenteric arteries	Koop <i>et al</i> , 2003, 2005
<i>Tight junctions</i>		
Claudin 5	No morphological alterations, but a size-selective loosening of the blood–brain barrier	Nitta <i>et al</i> , 2003
Occludin	No vascular phenotype	Saitou <i>et al</i> , 2000
JAM-A	No vascular phenotype in the embryo; in the adult, defective angiogenic and inflammatory responses	Cera <i>et al</i> , 2004 Cooke <i>et al</i> , 2006
JAM-C	No vascular phenotype in the embryo, but altered response to permeability and inflammatory stimuli	Gliki <i>et al</i> , 2004 Orlova <i>et al</i> , 2006
ESAM	No morphological phenotype, but decreased angiogenesis and permeability response in the adult	Ishida <i>et al</i> , 2003 Wegmann <i>et al</i> , 2006
ZO1	Defective chorioallantoic fusion and yolk-sac angiogenesis	Katsuno <i>et al</i> , 2008
ZO2	Lethality before the onset of vascular development	Xu <i>et al</i> , 2008
ESAM, endothelial-cell-selective adhesion molecule; JAM, junctional adhesion molecule; N-cadherin, neuronal cadherin; PTP, protein tyrosine phosphatase; RPTP μ , receptor-like protein tyrosine phosphatase μ ; VE, vascular endothelial; ZO, zonula occludens.		

concentration and inducing its dimerization. Free α -catenin dimers can, in turn, reduce actin branching and increase filament bundling (Yamada *et al*, 2005; Weis & Nelson, 2006). Another important partner is p120^{cas}, which binds to a juxtamembrane domain of VE-cadherin (Fig 1A). p120^{cas} is an inhibitor of Rho family GTPases (Anastasiadis *et al*, 2000), and also modulates VE-cadherin membrane stability and endocytosis (Xiao *et al*, 2005). In endothelial cells, which do not have desmosomes, plakoglobin can also link VE-cadherin to vimentin-based intermediate filaments through desmoplakin, forming junctions of a different type, which are known as complexus adhaerentes (Valiron *et al*, 1996; Kowalczyk *et al*, 1999; Hammerling *et al*, 2006). In addition to being components of the AJ complex, β -catenin, plakoglobin and p120^{cas} can translocate to the nucleus and regulate gene expression (Nelson & Nusse, 2004; Brembeck *et al*, 2006; Park *et al*, 2006; Reynolds, 2007).

Conditional inactivation of β -catenin in the endothelium using the Cre/loxP system resulted in defective vascular patterning and vessel integrity, and caused early embryo lethality (Cattellino *et al*, 2003). Blind-ending vessels and lacunae-like bifurcations were

found in β -catenin-null animals, the vessels of which were characterized by an inconsistent lumen and frequent haemorrhages (Cattellino *et al*, 2003). Interestingly, the absence of β -catenin resulted in a rearranged organization of the junctional complex. Whereas the level of α -catenin was markedly reduced, both the expression level and the junctional localization of desmoplakin were strongly increased, indicating a switch from actin-based AJs to vimentin-based complexus adhaerentes (Cattellino *et al*, 2003). Similarities between β -catenin-null embryos and the knockout of the Wnt receptor frizzled 5 (Ishikawa *et al*, 2001) indicate that some of the features of null embryos might be owing to the inhibition of Wnt signalling. In agreement with this, the lack of β -catenin transcriptional activity was shown to abolish the epithelial–mesenchymal transformation of endocardial cells and thereby heart-cushion formation (Liebner *et al*, 2004).

Total knockout of plakoglobin resulted in embryo lethality owing to severe heart defects that were related to a reduced number of desmosomes in the myocardium (Ruiz *et al*, 1996; Bierkamp *et al*, 1996). However, the ablation of plakoglobin was

not observed to affect endothelial cells, possibly because the lack of plakoglobin at AJs is compensated by β -catenin. In contrast to plakoglobin, no compensatory counterpart seems to exist for desmoplakin, as desmoplakin-deficient mice show vascular problems such as a reduced number of capillaries and haemorrhages (Gallicano *et al*, 2001).

Adherens-junction-associated protein tyrosine phosphatases

Several cytoplasmic and transmembrane PTPs associate with the AJ complex. VE-PTP is the only one that is restrictively expressed in the endothelium (Fachinger *et al*, 1999; Baumer *et al*, 2006; Dominguez *et al*, 2007). VE-PTP has been shown to associate with, and dephosphorylate, VE-cadherin (Nawroth *et al*, 2002). Deletion of VE-PTP from the plasma membrane or knockout of the *VE-PTP* gene causes abnormal vessel development after embryonic day 8.5 (E8.5), growth retardation and halted development at E9.0 (Baumer *et al*, 2006; Dominguez *et al*, 2007). After forming the initial primitive vascular plexus, the vascular network of VE-PTP-null embryos failed to remodel and deteriorated (Baumer *et al*, 2006; Dominguez *et al*, 2007). These defects could be a result of VE-cadherin hyperphosphorylation.

CD148 (DEP1/PTP η) is not restrictively expressed in the endothelium, but is abundant in the endothelial cells of the arterial and capillary vessels of several organs (Takahashi *et al*, 2003). The inhibition of CD148 activity through a catalytically inactive construct resulted in incorrect vascular development. The dorsal aorta was narrowed, whereas the intersomitic vessels were enlarged, and several abnormalities were observed in the heart. Similar to VE-PTP-deficient mice, yolk-sac vessels in CD148-mutant mice showed no signs of remodelling, but merged together and formed large hyperfused vessels with discontinuous endothelial cell layers. In contrast to VE-PTP-deficient mice, increased endothelial cell proliferation was observed in the yolk-sac vessels of CD148-deficient animals (Takahashi *et al*, 2003). A correlation between decreased CD148 expression and increased endothelial cell proliferation has been established *in vitro* in cultured endothelial cells. CD148 was shown to dephosphorylate VEGF receptor 2 (VEGFR2) when associated with the VE-cadherin/ β -catenin complex, thereby reducing VEGF-stimulated mitogen-activated protein (MAP) kinase activation and cell proliferation (Lampugnani *et al*, 2003). Furthermore, using a bivalent antibody against the ectodomain of CD148, growth arrest could be induced in endothelial cells *in vitro* and angiogenesis could be blocked *in vivo* (Takahashi *et al*, 2006).

The receptor-like PTP μ (RPTP μ) is expressed by a wide range of cell types, but is largely present at endothelial junctions *in vivo* (Bianchi *et al*, 1999), with predominant expression in arteries and in the capillaries of continuous endothelium (Koop *et al*, 2003). Although RPTP μ might associate with, and dephosphorylate, p120^{cas} (Feiken *et al*, 2000) and VE-cadherin (Sui *et al*, 2005) *in vitro*, total knockout of the *RPTP μ* gene resulted in viable and fertile mice with no obvious phenotype (Koop *et al*, 2003). Detailed investigation of RPTP μ -deficient mice showed that flow-induced dilation in mesenteric arteries was impaired, although blood pressure was unaffected (Koop *et al*, 2005).

Tight-junction components

As a result of organ-specific requirements, there is considerable variability in the organization of TJs along the vascular tree. Hence, TJs are well developed in brain vessels—where they contribute to the

blood–brain barrier—and in arteries, whereas they are less organized in veins and organs that are characterized by a high rate of transport (Bazzoni & Dejana, 2004). Claudins are transmembrane proteins that constitute a large gene family with 24 known members that form the backbone of TJ strands (Furuse & Tsukita, 2006). Several claudins are expressed in endothelial cells, such as claudin 1, claudin 3, claudin 5 and claudin 12, but only claudin 5 is endothelial-cell-specific (Morita *et al*, 1999). Inactivation of the claudin 5 gene in mice did not morphologically alter the vascular network or the ultrastructural appearance of the TJs (Nitta *et al*, 2003). However, claudin-5-deficient pups died within 10 h of birth owing to a size-selective loosening of the blood–brain barrier against molecules that were <800 Da. The transmembrane protein occludin is structurally similar to claudins and is incorporated into claudin-based strands. Occludin is present in endothelial cells, particularly in the brain (Hirase *et al*, 1997). However, no effects on vascular morphology or blood–brain barrier permeability have been reported in mice that lack occludin (Saitou *et al*, 2000).

JAM-A and its related family members—JAM-B, JAM-C, endothelial-cell-selective adhesion molecule (ESAM) and coxsackievirus and adenovirus receptor (CAR)—are transmembrane glycoproteins that associate with TJ strands, but do not constitute the strands *per se* (Itoh *et al*, 2001; Bazzoni & Dejana, 2004; Weber *et al*, 2007). All JAM family members and ESAM are expressed in endothelial cells; however, inactivation of their respective genes in mice did not affect the development of the vascular system in the embryo (Ishida *et al*, 2003; Cera *et al*, 2004; Cooke *et al*, 2006; Wegmann *et al*, 2006; Orlova *et al*, 2006). In adult mice, all of these molecules have an important role in modulating leukocyte diapedesis through endothelial cells. Furthermore, the Matrigel plug assay showed a deficiency in the angiogenic response towards fibroblast growth factor 2 (FGF2) in both JAM-A-null and ESAM-null mice (Ishida *et al*, 2003; Cooke *et al*, 2006). In addition, a JAM-C-blocking antibody abolished angiogenic sprouting *ex vivo*, and reduced angiogenesis in the retina and in tumours (Lamagna *et al*, 2005).

ZO1 and ZO2 are closely related members of the membrane-associated guanylate kinase (MAGUK) family that localize at TJs in most tissues, including the endothelium (Bazzoni & Dejana, 2004; Katsuno *et al*, 2008). Ablation of ZO2 resulted in arrested development at E5.5 and lethality before the onset of vascular development (Xu *et al*, 2008). By contrast, embryos deficient for ZO1 developed normally until E8.5, and thereafter displayed growth retardation, defective chorioallantoic fusion and yolk-sac angiogenesis (Katsuno *et al*, 2008). Blood vessels in ZO1-null yolk sacs were characterized by defective remodelling and compartmentalization, as they expanded with only a few adhesion sites. Interestingly, JAM-A failed to localize at sites of cell adhesion, suggesting altered TJ organization (Katsuno *et al*, 2008).

Concluding remarks

Our knowledge of the molecular organization of endothelial cell–cell junctions has increased during the past years. Many new adhesive membrane proteins and intracellular partners have been identified and studied in different experimental settings both *in vitro* and *in vivo*. In most cases, AJ components are necessary for correct vascular morphogenesis, and TJ proteins are needed for the maintenance of endothelial barrier properties. However, many issues remain unsolved (Sidebar A). For instance, we know

Sidebar A | In need of answers

- (i) How are cell-to-cell junctions organized during angiogenesis? Is it possible to stabilize endothelial junctions to limit vessel growth?
- (ii) Do adherens junctions influence tight-junction organization and vice versa?
- (iii) Endothelial cells express cell-specific members of large families of junctional adhesive proteins such as vascular endothelial-cadherin or claudin 5. Do these proteins transfer endothelial-specific signals?

only partly how different junctional structures cross-talk, and we do not know whether incorrect AJ assembly affects TJ organization. Moreover, the mechanisms that regulate the dynamic opening and closing of junctions, and the pathways through which junctions transfer intracellular signals, remain largely obscure.

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