Claudin-5 Controls Intercellular Barriers of Human Dermal Microvascular but not Human Umbilical Vein Endothelial Cells

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Objective—To assess the role claudin-5, an endothelial cell (EC) tight junction protein, plays in establishing basal permeability levels in humans by comparing claudin-5 expression levels in situ and analyzing junctional organization and function in 2 widely used models of cultured ECs, namely human dermal microvascular (HDM)ECs and human umbilical vein (HUV)ECs.

Methods and Results—By immunofluorescence microscopy, ECs more highly express claudin-5 (but equivalently express vascular endothelial-cadherin) in human dermal capillaries versus postcapillary venules and in umbilical and coronary arteries versus veins, correlating with known segmental differences in tight junction frequencies and permeability barriers. Postconfluent cultured HDMECs express more claudin-5 (but equivalent vascular endothelial-cadherin) and show higher transendothelial electric resistance and lower macromolecular flux than similarly cultured HUVECs. HDMEC junctions are more complex by transmission electron microscopy and show more continuous claudin-5 immunofluorescence than HUVEC junctions. Calcium chelation or dominant negative vascular endothelial-cadherin overexpression decreases transendothelial electric resistance and disrupts junctions in HUVECs, but not in HDMECs. Claudin-5 overexpression in HUVECs fails to increase transendothelial electric resistance or claudin-5 continuity, whereas claudin-5 knockdown in HDMECs, but not HUVECs, reduces transendothelial electric resistance and increases antibody accessibility to junctional proteins.

Conclusion—Claudin-5 expression and junctional organization control HDMEC and arteriolar-capillary paracellular barriers, whereas HUVEC and venular junctions use vascular endothelial-cadherin. (Arterioscler Thromb Vasc Biol. 2013; 33:00-00.)

Key Words: endothelial cell ■ heterogeneity ■ microvessel ■ permeability ■ tight junction

Microvascular endothelium forms the primary barrier for the bidirectional exchange of blood gases, fluid, soluble nutrients, and waste between blood and tissues. Basal microvessel permeability varies segmentally between lowest in arteries, intermediate in continuous capillaries (although very low within the capillaries of the central nervous system; CNS), and greater in postcapillary venules.1–4 Basal permeability is a function of surface area (vast in capillary beds), flow rate (slowest though capillaries and post capillary venules), and physical properties of the lining endothelium.5 Routes for molecular exchange across microvascular endothelium can be paracellular, that is between adjacent endothelial cells, (ECs) or transcellular via vesicles, or vesicular-vacuolar organelles.6 The principal difference in basal permeability between the capillaries and the postcapillary venules into which they empty is thought to reside in physicostructural properties of EC junctions.7–9

Interendothelial junctions may regulate permeability through 2 different types of cytoskeleton-anchored protein complexes, adherens junctions (AJs) and tight junctions (TJs). Both are composed of transmembrane molecules whose extracellular domains form homophilic attachments that join adjacent ECs. AJs are organized around the transmembrane molecule vascular endothelial (VE)-cadherin and require calcium ions for stability.10–15 The intracellular portion of VE-cadherin interacts with cytoplasmic proteins (p120, α- and β-catenin, and plakoglobin) that anchor VE-cadherin to the cytoskeleton.16-21 In epithelial cells, TJ formation but not maintenance requires expression of E-cadherin, the primary cadherin in this cell type.22 VE-cadherin seems to play a similar role in ECs.16 In epithelial cells and in ECs, TJs are organized around claudin proteins, the most abundant of which in ECs is claudin-5.23–25 All claudins are tetramembrane spanning proteins oriented with both their amino and
carboxy termini in the cytosol, resulting in the formation of 2 extracellular loops that control selectivity and adhesion. Homophilic claudin-5 interactions at TJs oppose adjacent ECs more closely than does VE-cadherin at AJs. Unlike VE-cadherin extracellular adhesion, claudin-5-mediated adhesion is calcium-independent. A conserved carboxy terminus YV-amino acid motif within the intracellular portions of claudins associates with PSD95/Disc Large and Zona occludens (PDZ) domain proteins, such as ZO-1, -2, and -3; claudin-5, in particular, associates with the multi-PDZ domain protein-1. Such interactions that link claudins to the actin cytoskeleton appear in transmission EM as a diffuse band of dense cytoplasmic material, also referred to as a cytoplasmic plaque. In epithelial cells, TJs are spatially segregated into a continuous ring between adjacent cells at the apical-lateral border that is separated from AJs. In contrast, EC TJs do not form a continuous ring and spatially intermix with AJs, although it is unclear whether TJ and AJ molecules in ECs physically associate. Selective transcellular permeability (perselectivity) in epithelial barriers is mediated by their TJs. TJ abundance in ECs is greatest in arterioles, intermediate in postcapillary venules, and least organized in continuous capillaries, and least organized in postcapillary TJs. TJ abundance in ECs is greatest in arterioles, intermediate (permselectivity) in epithelial barriers is mediated by their TJs. TJ abundance in ECs is greatest in arterioles, intermediate (permselectivity) in epithelial barriers is mediated by their TJs. TJ frequency generally correlates inversely with TJs in EC throughout the vascular system, we hypothesize that differences in claudin-5 expression might determine the barrier strength of EC derived from different segments of the human peripheral vasculature, a role described only in EC derived from brain microvessels. Here, we report that differences in claudin-5 expression levels correlate with the barrier strength formed by human ECs in situ by an analysis comparing umbilical cord large vessels to skin microvessels. We also report differences in the level of claudin-5 expressed by cultured human dermal microvascular (HDM) ECs that form high resistance barriers versus cultured human umbilical vein (HU) ECs that do not. Claudin-5 expression is required to limit paracellular permeability in HDM EC monolayers, whereas VE-cadherin performs this function in monolayers of HUVECs. However, claudin-5 overexpression in HUVECs fails to produce high resistance barriers, consistent with our observation that HDMECs and HUVECs organize claudin-5 differently at their respective junctions. These results establish HDMECs as a better model than HUVECs for analysis of human microvascular endothelial TJs.

Methods

For detailed Methods please see online-only Data Supplement.

Confocal and Epifluorescence Immunomicroscopic Analyses of Human Tissues

Specimens of normal human skin, umbilical cord, or epicardium were prepared as frozen sections and immunostained using methods and antibodies described in the online-only Data Supplement. For all microscopy procedures, during image acquisition, intensity levels were calibrated to the most intense signal and kept constant for a given experiment.

EC Cultures

HDMECs in normal adult human skin from anonymized donors were isolated as described. Serially passaged HDMECs uniformly express the lymphatic markers Prox-1 and Podoplanin (unpublished data) and concomitantly express E-selectin in response to tumor necrosis factor, a characteristic feature of blood vascular ECs. HUVEC cultures were established as previously described, then weaned gradually into the same EGM2-MV medium as HDMECs and used between passage 4 and 6. For all experiments in this study, each EC type was seeded onto human plasma fibronectin-coated substrates at 2/3 confluence attained visual confluence at or before 24 hours post-plating (designated as day 0 postvisual confluence).

DNA Constructs and Transductions

An IL2R-VE retroviral construct was assembled from cDNA of the IL2R-VE-cadherin fusion protein, consisting of the human IL2Rα (CD25) extracellular and transmembrane domains fused to the human VE-cadherin cytoplasmic domain in a pCMV plasmid kindly provided by Dr Andrew Kowalczyk (Emory University). A human claudin-5 retroviral construct was assembled from human claudin-5 cDNA (clone ID 5242567 obtained from Open Biosystems) and sub-cloned into the retroviral vector pLZRS.CMV. A retroviral EGFP-claudin-5 construct was assembled from cDNA of an N-terminal EGFP-sequence fused to the full length human wild-type cDNA sequence of claudin-5 within the pEGFP.C1-vector (Clontech).

Human GIPZ lentiviral short hairpin RNA constructs used for lentivirus knockdown were obtained as glycerol stabs from Open Biosystems.

FACS Analysis and Immunoblotting

For FACS analyses of junctional molecule expression, ECs first cultured to day 3 postvisual confluence were immunostained with or without permeabilization. For immunoblot analyses, cultured ECs scrape-harvested on ice into Laemmli buffer were analyzed as described.

Confocal and Epifluorescence Immunomicroscopic Analyses of Cells

Immunomicroscopic analyses were performed on ECs on fibronectin-coated glass cover slips at day 3 postvisual confluence unless indicated.

Electron Microscopy

ECs were seeded onto fibronectin-coated high-density 0.4-μm pore size 6-well format cell culture inserts (BD Biosciences) and on day 3 postvisual confluence adhered to them for EM analysis. Juxtapositions of plasma membrane processes from neighboring EC (overlap regions in Table 1) were scored as tongue-in-groove structures where at least
Table 1. Ultrastructural Features of Day 3 Postvisual Confluent HDMEC and HUVEC Monolayers

<table>
<thead>
<tr>
<th>EC Type</th>
<th>Tongue-in-Groove Structures per Overlap Region ±SEM</th>
<th>Tight Juncions per Overlap Region ±SEM</th>
<th>Maximum TEER, ohms ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDMEC*</td>
<td>1.77±0.25</td>
<td>4.94±0.67</td>
<td>3939±122</td>
</tr>
<tr>
<td>HUVEC†</td>
<td>0.29±0.13</td>
<td>1.53±0.19</td>
<td>1478±36</td>
</tr>
</tbody>
</table>

Unpaired 2-tailed t test Significant: *P<0.0001 †P<0.0001 Significant: P<0.0001

EC indicates endothelial cell; HDMEC, human dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; and TEER, transendothelial electric resistance. *Pooled data from 3 different isolates. †Pooled from 2 different isolates.

Transendothelial Flux and TEER Measurements

For transendothelial flux measurements, ECs were seeded onto fibronectin-coated 0.4-μm pore 24-well size cell culture inserts (BD Falcon). FITC-dextrans (either 3 kD or 70 kD, from Invitrogen) suspended in EBM basal medium (base medium for EGM-2MV, Lonza) were added into the apical chamber. Barrier function of ECs cultured on fibronectin-coated 8W10E+ gold electrode 8-chamber slides was also assessed by electrical cell-substrate impedance sensing (Applied BioPhysics).46

Calcium Chelation Assay

The effects of calcium chelation on transendothelial electric resistance (TEER), intercellular gap formation, or displacement of VE-cadherin from EC junctions were assessed by replacing complete medium with warmed serum-free (1% BSA) EBM basal medium; basal medium/BSA supplemented with 4 mmol/L EGTA; basal medium/BSA/EGTA supplemented with 16 mmol/L Ca++; or basal medium/BSA/EGTA supplemented with 16 mmol/L Mg++.46

Results

Claudin-5 Expression Correlates With Segmental Permeability Differences

We first tested the hypothesis that claudin-5 expression by ECs in the peripheral vasculature correlates with known segmental differences in permeability in 3 human tissues. Umbilical cord is readily available as discarded material and widely used as a source for isolation and culture of human ECs. In the large vessels of the umbilical cord, claudin-5 was expressed at markedly higher levels in arterial ECs than in venous ECs. In contrast, the AJ protein VE-cadherin was more comparably expressed by the same vessel types (Figure 1A–C). However, because umbilical vessels may differ from other vessel beds with regard to claudin-5 expression owing to their exceptional oxygen and pressure levels when carrying blood between placenta and the fetus, we performed additional staining experiments of human adult cardiac vessels. Claudin-5 is expressed at higher levels in human coronary artery than in human coronary vein, whereas in contrast, VE-cadherin is expressed at similar levels in both vessel types. Thus our findings that the relative variability of claudin-5 expression versus the relative constancy of VE-cadherin expression are not restricted to umbilical vessels (please see online-only Data Supplement Figure I), which also displays immunostained umbilical vessels as confocal microscopy images. In the superficial vascular plexus of human skin, the capillaries are readily distinguished from other microvessels by their unique anatomic position as vascular loops located within the dermal papillae near epidermal rete ridges, whereas the paired arterioles and venules that run parallel to the epidermal surface are located more distal to the rete, and differ by the much greater degree of investment of arterioles compared with venules by SMA-expressing mural cells.37-50 Within the papillary dermis, claudin-5 expression was highest in arterioles, intermediate in capillaries, and lowest in venules. Unlike claudin-5, VE-cadherin and in addition to the scaffold protein ZO-1 were found expressed at largely comparable levels by ECs in all 3 types of dermal microvessels (Figure 1D–1H and online-only Data Supplement Figure II). These observations are consistent with the hypothesis that claudin-5 expression controls basal paracellular vascular permeability in human peripheral tissues just as it does in the microvessels of the mouse CNS.

Barrier Properties, Claudin-5 Expression, and Junctional Organization of Microvascular ECs

We next used ECs cultured from these tissues to study the barrier function of EC junctions. We first quantified the barriers formed by these 2 cell types using electrical cell-substrate impedance sensing. In EGM2-MV medium, HUVEC monolayers achieved a barrier strength on a fibronectin-coated 8W10E+ electrode array of 1721±47 ohms and, under identical culture conditions, HDMEC monolayers produce a maximum barrier of 3837±78 ohms (in 8 independent experiments), or a roughly 2-fold higher TEER. HUVEC monolayers typically attain maximum barrier integrity soon after HUVEC-HUVEC contacts are initially established as assessed by visual inspection, (designated as day 0 postvisual confluence). In contrast, HDMECs form a barrier of comparable strength to that of HUVECs at visual confluence but then further increase their barrier over the next 3 to 5 days before reaching a plateau at the higher level of TEER described above (Figure 2A). The TEER time courses and maximal TEER levels of both EC cell types were similar whether the electrodes were coated with human fibronectin or with human collagen IV (please see online-only Data Supplement Figure III) and all subsequent studies in this report used fibronectin-coated surfaces. Consistent with a higher level of TEER, HDMEC monolayers limited transendothelial flux of 3000 or 70 000 D FITC-dextrans compared with HUVECs (Figure 2B). We also examined whether HDMEC limits transendothelial flux via a more effective paracellular barrier or via slower vesicular transport by comparing transendothelial flux at 37°C, a temperature that permits endocytosis, and at 4°C, a temperature that does not. These measurements showed that differences in flux of 3 kDa FITC-dextran across HDMEC and HUVEC monolayers were similar at both temperatures through 6 hours, when the concentration gradient between the upper and lower transwells still remained far from equilibrium.
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(please see online-only Data Supplement Figure IV), strongly suggesting that the difference among EC types is because of differences in their paracellular barriers and independent of vesicular transport.

To further analyze the intercellular junctions that formed in culture, we examined postconfluent monolayers of HDMECs and HUVECs by transmission EM. In microscope fields displaying regions of overlap by adjacent ECs, HDMECs formed multilayered protrusions that interdigitated in a tongue-in-groove fashion, creating a labyrinth-like paracellular path (Figure 2C, top). In contrast, HUVEC junctions have more simple topologies of adjacent cells that simply overlap (Figure 2D, top). Tongue-in-groove structures in overlap regions of HDMECs are 6-fold greater than in HUVEC monolayers, correlating with increased barrier formation by HDMECs (please see Table 1). In both EC types, we observed membrane approximations that seemed as kissing points near diffuse cytoplasmic densities, structures generally interpreted as TJs. Such TJ structures were significantly more numerous at HDMEC than HUVEC junctions and their number correlated with TEER measured on the same EC lines. These differences in the morphology of intercellular junctions likely underlie the observed differences in EC barrier properties.

To explore molecular differences at EC junctions, we compared the expression levels of claudin-5 and other EC junctional proteins between these 2 cultured EC types by FACS analysis and immunoblotting. On day 3 postvisual confluence, HDMECs uniformly express claudin-5 at levels more than twice high as HUVECs but express VE-cadherin (CD144) and ZO-1 at levels comparable to HUVECs as quantified by FACS (Figure 3A). Also by densitometric analysis of immunoblotting, claudin-5 expression on day 3 postvisual confluence is 2-fold greater in HDMECs than in HUVECs, and VE-cadherin is expressed at equivalent levels in both EC types (Figure 3B and 3C; normalized to β-actin). Occludin was more abundant in HUVEC and barely detectable in HDMEC. Interestingly, during 3 days at postconfluence, claudin-5 increased 3.6-fold in HDMEC (P<0.5), whereas VE-cadherin did not. Increases in claudin-5 expression levels correlate with the TEER increase noted for HDMEC monolayers, but
Despite considerable HUVEC expression of claudin-5 by day 3 postconfluence, HUVEC monolayers showed no increase in TEER (Figure 3C). By immunoblotting, ZO-1 expression did not change at postconfluence in both EC types (please see online-only Data Supplement Figure V).

We next used confocal fluorescence immunomicroscopy to characterize spatial patterns of claudin-5 and VE-cadherin expression at HDMEC and HUVEC junctions. In HDMECs on day 0 postvisual confluence, claudin-5 localization at junctions is minimal, but both the junctional localization and intensity of claudin-5 staining progressively increase, condensing by day 3 postconfluence to a thin but bright continuous band outlining the cell (Figure 4A). However in HUVECs, the staining pattern of claudin-5 remains sawtooth and discontinuous at many points along their borders through day 3 postvisual confluence (Figure 4B), never producing the continuous pattern of junctional staining characteristic of HDMECs. Localization of VE-cadherin to the cell junction, as detected with a goat antibody to an intracellular epitope in permeabilized cells, preceded the junctional localization of claudin-5 in both EC types (Figure 4A). However, in HDMECs that were not permeabilized, staining of VE-cadherin with antibody to an extracellular epitope decreased as the cells progressively tightened their barrier, suggesting reduced antibody access to the intercellular space (Figure 4C). This reduced staining of extracellular VE-cadherin epitopes was not observed in postconfluent HUVECs (Figure 4D) and similar changes in accessibility to extracellular epitopes of PECAM-1 were seen in postconfluent HDMECs but not HUVECs (data not shown).

Thus a progressive change in claudin-5 organization as well as expression at the intercellular junctions of HDMEC monolayers correlates with both an increase in TEER and a reduction in accessibility to the paracellular space.

Cadherins are necessary for formation of TJs but may not be necessary for their maintenance. Therefore, we tested the role of VE-cadherin in established HDMEC and HUVEC monolayers by overexpression of a dominant negative fusion protein in which the VE-cadherin intracellular cytoplasmic domain is fused to the extracellular and transmembrane domains of interleukin-2 receptor α chain (CD25). The VE-cadherin intracellular domain of this construct (IL2R-VE) displaces...
We confirmed by immunoblotting, first with an anti-IL2Rα antibody (that only recognizes expression of IL2R-VE) and then with goat polyclonal anti-VE-cadherin (which recognizes both IL2R-VE and endogenous VE-cadherin) that IL2R-VE was expressed at the expected size and at comparable levels in both FACSorted EC lines (Figure 5A and 5B). Both HDMECs and HUVECs overexpressing this construct downregulate surface expression of endogenous VE-cadherin without affecting levels of claudin-5 expression as assessed by FACS analysis.21

permeability in immortalized HMEC-1 cell monolayers.17,44 We confirmed by immunoblotting, first with an anti-IL2Rα antibody (that only recognizes expression of IL2R-VE) and then with goat polyclonal anti-VE-cadherin (which recognizes both IL2R-VE and endogenous VE-cadherin) that IL2R-VE was expressed at the expected size and at comparable levels in both FACSorted EC lines (Figure 5A and 5B). Both HDMECs and HUVECs overexpressing this construct downregulate surface expression of endogenous VE-cadherin without affecting levels of claudin-5 expression as assessed by FACS analysis.21
(unpublished data). IL2R-VE overexpression decreased TEER by 60% in HUVEC monolayers, but in postconfluent HDMEC monolayers merely delayed reaching the maximal level of TEER, which was unchanged (Figure 5C). Consistent with the effect of dominant negative VE-cadherin overexpression on TEER, the junctions of transduced HUVECs but not HDMECs were disrupted and formed gaps (Figure 5D). We also exploited the fact that VE-cadherin-based AJ adhesion is strongly calcium-dependent, whereas with claudin-based TJ adhesion is not \(^{11,30}\) by replacing EGM-2MV medium on EC monolayers grown on electrical cell-substrate impedance sensing electrodes with serum- and growth factor-free basal medium containing 4 mmol/L EGTA (a 2-fold molar excess over medium [Ca\(^{++}\)]). Within seconds after calcium chelation, HUVEC TEER fell by 75%, whereas HDMEC TEER persisted at normal levels through 1 hour after which their barriers gradually weaken (online-only Data Supplement Figure VI and data not shown). The fall in TEER correlated with the development of visible gaps between adjacent HUVECs, a finding not observed in HDMEC monolayers subjected to calcium chelation. VE-cadherin in HUVECs, but not in HDMECs, was displaced from intercellular junctions by EGTA-containing basal medium as judged by epifluorescence immunofluorescence microscopy. Overall, these observations suggest that postconfluent HDMEC junctions are far less dependent than HUVEC on VE-cadherin for their integrity and function.

**Claudin-5 Is Required for HMDEC, Not HUVEC Barrier Properties**

To further contrast the role of claudin-5 in HUVECs, we transduced these cells with a drug selectable retrovirus encoding N-terminally tagged EGFP-claudin-5 (EGFP-claudin-5) or EGFP alone (negative control). After selection, HUVECs strongly expressed EGFP-claudin-5 or EGFP (control) by FACS analysis. Immunoblotting confirmed HUVEC expression of EGFP-claudin-5 protein at the expected apparent molecular weight relative to endogenous claudin-5. Despite EGFP-claudin-5 overexpression at HDMEC-like levels, fluorescence in HUVECs localized to junctions only in the discontinuous, sawtooth pattern characteristic of endogenous claudin-5 in this EC type. Although many junctions in HDMECs overexpressing EGFP-claudin-5 also showed a disrupted fluorescence pattern, some junctions in postconfluent monolayers of transduced HDMEC monolayers showed patterns of EGFP fluorescence.

**Figure 5.** Effects of dominant negative vascular endothelial (VE)-cadherin protein overexpression. A, FACS analyses of interleukin (IL)-2Ra immunostaining of human dermal microvascular endothelial cells (HDMECs) and human umbilical vein endothelial cells (HUVECs) transduced with plasmid LZRS empty vector control (left) or with the otherwise identical retroviral vector containing an IL2R-VE insert (right). The IL2R-VE transductants were selected previously by positive FACS sorting with anti-IL-2Ra. Specific staining (filled histograms) with APC-conjugated anti-IL2R \(\beta\) is compared with staining with isotype control from 3 independent experiments. *\(P=0.04\) by unpaired 2-tailed \(t\) test. B, Immunoblotting of cell lysates of the same transduced cell cultures shown in A. Top: Protein lysates from empty LZRS vector control and IL2R-VE-cadherin-transduced HDMECs (lanes 1 and 2) or HUVECs (lanes 3 and 4) analyzed with rabbit anti-VE-cadherin. Arrow: band representing endogenous VE-cadherin; Arrowhead: IL2R-VE-cadherin fusion protein. The same protein lysates were analyzed by immunoblotting with goat-anti-IL2R \(\beta\) (middle) or anti-\(\beta\)-actin (bottom). Numbers indicate densitometric analyses of the immunoblot data normalized to expression of \(\beta\)-actin. C, TEER development in HDMECs and HUVECs transduced with dominant negative IL2R-VE-cadherin. Over a 6-day time course starting at postconfluence, TEER levels did not differ between control and IL2R-VE-transduced HDMECs (NS, not significant by 1-way ANOVA with Bonferonni correction and not significant by 2-tailed test on day 6) but were consistently different between control and IL2R-VE-transduced HUVEC \((P<0.05\) by 1-way ANOVA with a Bonferonni correction and \(P=0.0005\) by 2-tailed test on day 6). Mean TEER values were analyzed from 4 independent experiments. D, Immunofluorescence staining of FACS-sorted EC for VE-cadherin (with goat anti-VE-cadherin to an intracellular epitope, left), and for claudin-5 (middle) on permeabilized cells and for CD31PECAM-1 (right, but on intact cells) comparing empty vector control and IL2R-VE-transduced HDMECs and HUVECs at day 3 postvisual confluence. Arrows show where IL2R-VE-transduced HUVECs but not HDMECs form gaps. Scale bar =18 \(\mu\)m. The experiments shown in Figure 5 are representative of 3 sets of transduced endothelial cell lines analyzed with similar results.
that was continuous, similar to the pattern of immunostaining of endogenous claudin-5 in this cell type (please see online-only Data Supplement Figure VII). Free EGFP did not localize to junctions in either cell type but unexpectedly reduced the level of TEER. We therefore compared the functional effects of overexpressing untagged claudin-5 versus a control retroviral vector minus an insert. Claudin-5 overexpression in HUVECs was confirmed by immunoblotting and by increased immunostaining at junctions that, like EGFP-claudin-5, displayed patterns that were discontinuous and sawtooth. Overexpression of claudin-5 to levels seen in HDMECs did not increase TEER in postvisual confluent HUVECs but did inhibit gap formation between cells in response to chelation of calcium (please see online-only Data Supplement Figure VIII; the intercellular space after EGTA treatment corrected for medium control in μm² ± SEM was 2610±163 for claudin-5-transduced HUVEC versus 7718±685 for control-transduced HUVEC, a statistically significant difference \( t = 0.002 \) by a 2-tailed \( t \) test). Thus, increased claudin-5 expression is insufficient to form high resistance paracellular junctions in HUVECs, probably because HUVECs fail to organize claudin-5 in the manner observed in HDMECs.

We next tested whether endogenous claudin-5 expression is necessary for barrier functions in ECs by means of lentivirus-mediated shRNA knockdown. Two different lentiviral shRNA constructs targeting claudin-5 mRNA effectively reduced claudin-5 protein expression in HUVECs and HDMECs without affecting protein expression of VE-cadherin (Figure 6A). The extent of claudin-5 knockdown correlated with TEER decreases in HDMEC and, with the most effective shRNA vector HDMEC, TEER attained only one-half that of control-transduced HDMECs on day 4 postconfluence (Figure 6B). Claudin-5 shRNA knockdown also restored access to intercellular junctions between postconfluent HDMECs, allowing for bright staining of extracellular epitopes of VE-cadherin (Figure 6C) or of PECAM-1 (data not shown). In contrast, claudin-5 knockdown had no measurable effect on TEER levels in HUVECs (Figure 6B). ShRNA knockdown of the TJ protein, occludin which gradually localizes to junctions and seems to increase expression from day 0 to day 3 postconfluence in HUVECs, did not affect HUVEC TEER (please see online-only Data Supplement Figure IX). Thus claudin-5 expression in HDMECs seems essential for the characteristics that distinguish HDMEC junctions from those of HUVECs but plays no apparent role in HUVEC junctions.

**Discussion**

In our study, in situ expression of claudin-5 in human vascular EC, the signature protein component of endothelial TJs, correlates both with historically established variations of vascular EC barrier function to blood molecules and with the presence of TJs observed by EM, being higher in umbilical artery than vein and higher in arterioles and the continuous capillaries than in the venules of the dermal superficial vascular plexus. This suggested the simple hypothesis that variations in claudin-5 expression would correlate with TJ formation and with less permeable barriers in cultured human ECs from the same tissues. Indeed, we found that cultured HDMECs express claudin-5 at higher levels and form monolayers with reduced macromolecular flux and 2-fold higher levels of TEER than cultured HUVECs. We directly tested the importance of claudin-5 in HDMECs by shRNA knockdown, which resulted in reduced TEER and increased access of antibodies to the intercellular junctions. Furthermore, the barrier function of postconfluent HDMECs, but not of HUVECs, attained a state after several days that seemed independent of VE-cadherin as shown by the limiting effects on TEER and gap formation of both calcium chelation and overexpression of a dominant negative form of VE-cadherin. Moreover, as the HDMEC barrier progressively increased at postconfluence, there was a parallel increase in HDMEC expression of claudin-5. However, claudin-5 expression also increased in postconfluent HUVECs, and these cultures failed to increase their barrier to electric current. TEER in HUVEC cultures was also unaffected by either knockdown or by overexpression of claudin-5, although HUVEC that overexpressed claudin-5 did acquire a HDMEC-like resistance to intercellular gap formation induced by chelation of calcium. We attribute the difference in barrier formation between HDMECs and HUVECs to the manner in which endogenous or overexpressed claudin-5 is organized by these 2 cell types. Specifically, claudin-5 in HDMECs coalesced over time into a continuous and intensely focused staining at regions of HDMEC intercellular contact that is distinctly different from the sawtooth, discontinuous distribution of claudin-5 at the contact points formed by adjacent HUVECs. Junctions also seem more complex in HDMEC than HUVEC monolayers by transmission EM. Specifically, in HDMECs, we observed higher density of TJs identified as kissing points and more frequent interdigitating membrane tongue-in-groove morphologies than in HUVECs. Such interdigitating junctions have been observed in vivo and correlate with the impermeability to Evans Blue dye. These data demonstrate that phenotypic differences in the intercellular junctions among human EC types that correlate with permeability are represented in cultured HDMECs and HUVECs.

The term “junctional maturation” has been used to describe time-dependent reorganization in the barriers that form in epithelial cells and a similar process has been observed to occur in the CNS and in the brain-derived ECs in vitro. Our data suggest that cultured HDMECs undergo a claudin-5-dependent TJ maturation, contributing both to the progressive increase in TEER and to the progressive exclusion of antibodies from the interjunctional space. Although HDMECs continue to divide, increasing cell numbers at postconfluence, this change is insufficient to account for the observed maturation of their junctions because HUVEC similarly divide and increase in cell number without showing junctional maturation. Furthermore, the junctional maturation of HDMECs must depend on more than simply transporting claudin-5 to plasma membrane regions because both HDMECs and HUVECs localize claudin-5 to regions of EC-EC membrane contact. The failure of claudin-5 to influence the barrier formed by cultured HUVECs suggests that EC proteins other than claudin-5 must also contribute to barrier formation, perhaps, by organizing claudin-5 into the structures we observe as tight, continuous bands in cultured HDMECs. Junction maturation requires coordinated expression, localization, and interaction of transmembrane proteins, such as VE-cadherin or claudin-5.
with linker molecules, cytoskeleton adaptor molecules, and interactive enzymes that gradually assemble into a cytoplasmic plaque that anchors an AJ or TJ complex to the actin cytoskeleton.\textsuperscript{19,34,38,57,58} Proteins that help link claudins to the EC cytoskeleton include ZO-1, -2, or -3 and as described for claudin-5, multi-PDZ domain protein-1.\textsuperscript{31–33} Alternatively, HDMECs and HUVECs may differentially express other transmembrane TJ proteins that aid or inhibit cytoplasmic plaque assembly, respectively. For example, occludin, which is expressed at higher levels in HUVECs than HDMECs, could potentially interfere with claudin-5 organization, perhaps, by competing for the same interacting partner. However, occludin knockdown in HUVECs did not cause an increase in TEER, arguing against this interpretation (please see online-only Data Supplement Figure IX). Consistent with our observations that claudin-5-dependent barriers vary by EC type, others have observed claudin-5 overexpression leads to no increase\textsuperscript{59} or only a modest enhancement\textsuperscript{60} of barrier function by overexpression of claudin-5 in HUVECs. The modest enhancement of claudin-5 expression increase in HUVEC driven by lentivirus overexpression (described as massive)\textsuperscript{60} was likely greater than the amount of retrovirus-driven claudin-5 overexpression in our study. In contrast to the findings reported by Yuan et al,\textsuperscript{59} we found no effect of RNAi-mediated knock down of claudin-5 in HUVECs; the reason for this discrepancy is unclear, but we have found that transfection conditions used to introduce siRNA can affect barriers in a manner not seen with lentivirus transduction of shRNA. HUVEC barriers in our study and those of others seem mainly dependent on VE-cadherin at AJs.\textsuperscript{17} The barrier properties of HMEC-1, an immortalized EC line derived from human dermal foreskin that is often used as a model for microvascular EC\textsuperscript{61} also differs from the HDMECs we have studied in that the HMEC-1 barrier is, like HUVECs, VE-cadherin-dependent.\textsuperscript{44} Therefore, HDMECs may be more useful than HMEC-1s for studying capillary barriers.

Figure 6. Short hairpin RNA (shRNA) knockdown of claudin-5 in human dermal microvascular endothelial cells (HDMECs) and human umbilical vein endothelial cells (HUVECs). A. Top: Immunoblot analysis of claudin-5 expression in HDMECs (left) and HUVECs (right) transduced with nonsilencing lentivirus vector control (Non-Sil) or with claudin-5 shRNA clone V2LHS_171415 (CLS KD; top) or with a second claudin-5 shRNA, clone V2LHS_171412 (bottom) at day 4 postconfluent. Numbers indicate densitometric analyses of the immunoblot data normalized to expression of β-actin. B. Transendothelial electric resistance (TEER) levels in HDMECs or HUVECs stably transduced with shRNA pGIPZ clone V2LHS_171415 (pGIPZ-415) or clone V2LHS_171412 (pGIPZ-412) vs nonsilencing (Non-Sil) shRNA negative control-transduced ECs on day 3 postconfluence. Knockdown of claudin-5 in HDMECs significantly reduced TEER (P<0.0001 and P<0.005 by 2-tailed t test for GIPZ-415 and GIPZ412, respectively), whereas no significant TEER differences are observed in HUVECs transduced with either shRNA. TEER values shown are means from multiple experiments with pGIPZ-415 (n=5, 3) and with pGIPZ-412 (n=5, 5). C. Confocal fluorescence optical Z-sections of vector control (top) versus claudin-5 knockdown HDMEC (bottom) monolayers at day 4 postconfluent immunostained with mouse mAb BV6 to VE-cadherin extracellular epitopes in nonpermeabilized cells (arrows indicate differential antibody accessibility) and goat anti-VE-cadherin antibody to intracellular epitopes or rabbit anticlaudin-5 antibody in permeabilized cells. Scale bar =15 μm. One of 3 experiments with similar results.
to maintain cell junctions, in contrast to a very large number of articles that claim the opposite. Our data do not show that HDMEC barriers are completely VE-cadherin independent but are unequivocal that barriers formed by HDMEC are far less dependent on VE-cadherin than those of HUVEC.

Despite a common embryological origin and a shared number of features that lead to defining ECs as a distinct cell type, ECs adapt their morphological and functional features at distinct anatomical sites and among different vascular segments of the same tissue.62 HUVECs were the first and remain the most widely used model system to study ECs in culture. But as others have shown and we confirm here, junctional barriers in these cells are largely maintained by VE-cadherin-organized AJs, rather than claudin-organized TJs. They are a useful model for TJ-poor postcapillary venules13,17,63 but not for TJ-rich capillaries. At present there is no comparably well-accepted in vitro model for studying the distinct features of continuous capillaries possibly because microvascular ECs show greater tissue to tissue variation than do systemic large vessel ECs. Several investigators have concentrated on brain-derived ECs because they wish to study the unique properties of the blood–brain barrier. However, human material for primary cultures is limiting, provides variable or unstable phenotypes, and many such studies rely on immortalized cell lines.64,65 Furthermore, the formation of a CNS-like tight barriers by such cells often requires CNS-derived extrinsic factors like astrocyte-conditioned medium.66,67 The unique properties of the vasculature within the CNS also raises questions about whether findings using these cells can be extrapolated to peripheral microvessels. We have chosen to study HDMEC because we can readily compare findings made with this cell type in culture with cells in an accessible tissue, namely human skin. There are complications in this choice. Cultured HDMECs derive from a mixture of EC types originating in lymphatic microvessels, as well as from microvessels of the blood circulatory system. Our cultured HDMECs uniformly express lymphatic markers, Prox-1 and podoplanin, and also behave like blood vessel ECs by uniformly expressing E-selectin in response to tumor necrosis factor or interleukin-1β (unpublished data, M.S.K. and J.S.P.). Despite their heterogeneous origin, FACS analyses of our HDMECs in Figure 3 reveal a tight, uniform expression of the junctional molecules claudin-5, VE-cadherin, and ZO-1. Moreover, lymphatic vessels form barriers dependent on expression of the same TJ molecules as blood vessels.58,60 With regard to intercellular junctions, both EC types form similar structures at the level of transmission electron microscopy.70 In contrast to HDMEC used in our studies, Prox-1-negative dermal blood microvessel-derived EC (BEC purchased from Lonza) show decreased contact inhibition of migration so that confluent cells crawl over each other and form unstable intercellular barriers such that TEER decreases over time at confluence when cultured on human fibronectin or on collagen IV (MSK, unpublished observations). Such cells are thus less useful for the study of EC intercellular barriers. The decision of which EC culture model is best to study should rest on whether a distinct EC phenotype observed in situ is preserved in vitro, such as AJs in cultured HUVECs or TJs in cultured HDMECs. For example, to investigate the capillary leak syndrome associated with multiorgan failure in sepsis, HDMECs, which more faithfully represent the barrier properties of capillaries, would be a better model than HUVECs, which more closely resemble postcapillary venules.

It would be tempting to speculate that the sawtooth patterns we observed by immunofluorescence at HUVEC junctions for claudin-5 but not VE-cadherin may reflect differences in junctional ultrastructure observed by EM in which HUVEC junctions seemed to overlap and showed far fewer of the tongue-in-groove interdigitations than did HDMEC. In HDMEC, membrane interdigitations may provide a topology favoring the fine, continuous claudin-5 patterns we observed by confocal and epifluorescence microscopy, whereas in HUVEC, the simple overlapping of membranes from neighboring cells may distribute claudin-5 more sparsely, potentially explaining why relatively fewer TJs were counted per region of overlapping membranes in this EC type. However, in Figure 4, HUVECs seem no less adept than HDMECs at forming VE-cadherin-based AJs, which by virtue of the long VE-cadherin extracellular region consisting of 5 contiguous IgG domains may facilitate HUVEC-HUVEC interaction, despite the absence of tongue-in-groove interdigitations.

In conclusion, our data show that claudin-5 is expressed differentially and in a manner correlating with known TJ frequencies and barrier strengths of human blood vessels from outside the CNS, suggesting that claudin-5 could contribute to the segmentally arranged barrier heterogeneity of peripheral endothelium; claudin-5 expression is necessary but insufficient for establishing paracellular barriers in cultured EC; and claudin-5 expression, when continuously organized at TJs of postconfluent monolayers of HDMEC but not HUVEC, is critical for the maintenance of paracellular barriers in vitro independently of VE-cadherin.

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Disclosures
None.

References


Claudin-5 Controls Intercellular Barriers of Human Dermal Microvascular but not Human Umbilical Vein Endothelial Cells
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Antibodies and reagents

For immunohistochemistry and immunocytochemistry, we used polyclonal rabbit anti-claudin-5 (Invitrogen); goat anti-VE-cadherin to a C-terminal intracellular epitope (Santa Cruz Life Technologies); and mouse mAbs anti-CD31/PECAM-1, clone JC70A (DAKO) and anti-VE-cadherin, clone BV6 to an extracellular epitope¹ (Millipore). For FACS analyses we used mouse mAbs Alexa Fluor 488-conjugated anti-claudin-5, clone 4C3C2, FITC-conjugated anti-ZO1, clone ZO-1-1A12 (Invitrogen), Alexa Fluor 700-conjugated anti-VE-cadherin extracellular epitope, clone 16B1 (EBioscience), FITC-conjugated anti-human PECAM (CD31), clone WM59 and APC-conjugated anti-IL2Rα (CD25) chain, clone M-A251 (BD Pharmingen). For immunoblots, we used mouse mAbs anti-claudin-5 clone 4C3C2, anti-occludin clone OC-3F10 (Invitrogen), anti-GAPDH clone GAPDH-71.1 and anti-β-actin clone AC-74 (Sigma) as well as polyclonal goat anti-IL2Rα (R&D Systems) and rabbit anti-VE-cadherin (Cell Signaling).

Immunofluorescence microscopy of human tissues

Specimens of de-identified normal adult skin, neonatal umbilical cord and adult epicardial arteries and veins were obtained as discarded tissues under protocols approved by the Yale University Human Investigations Committee or the New England Organ Bank. Samples of these tissues were snap frozen in OCT (Sakura Finetek) in isopentane cooled to liquid nitrogen temperature and cryosectioned to a thickness of 5 μm. Tissue sections were subsequently fixed in 4% paraformaldehyde and incubated in blocking solution consisting of 1X TBS, 5% normal
donkey serum (Jackson ImmunoResearch Laboratories, Inc.), 5% BSA and 0.1% Triton X-100
(American Bioanalytical). Primary antibodies (rabbit anti-claudin-5, goat anti-VE-cadherin or
mouse anti-ZO-1) diluted in blocking solution to the final concentrations indicated were
incubated with the sections overnight at 4°C. After washing, sections were incubated for 1 hour
with donkey anti-rabbit, anti-goat or anti-mouse Alexa Fluor 647-conjugated secondary
antibodies (Invitrogen) diluted to a final concentration of 10 µg/ml in blocking solution. Control
sections (data not shown) were stained in a similar manner using rabbit, goat or mouse IgG
isotype controls (R&D Systems). The same sections were stained with FITC-conjugated Ulex
Europeus Agglutinin I (UEA-1; Sigma-Aldrich) to identify human ECs. Images of umbilical vessels
and skin microvessels in Figure 1 were acquired with an epifluorescence microscope (Axiovert
200M, Carl Zeiss) equipped with a HBO 100 digital camera using (for umbilical tissue) a FLUAR
10X magnification, air, numerical aperture 0.5 lens and (for dermal tissue) an ACHROPLAN 40 X
magnification, air, numerical aperture 0.6 lens. Confocal immunofluorescence images in
Supplemental Figures I and II were acquired with a spectral confocal microscope (Leica TCS SP5,
Leica Microsystems) equipped with acousto-optical filters, an acousto-optical beam splitter and
a Leica SP prism spectrophotometer detection system (using a HCX PL APO lambda blue 20X
magnification, oil, numerical aperture 0.7 IMM lens). In all cases, pairwise comparisons
between human umbilical artery and vein or between human coronary artery and vein or
among human skin arterioles, capillaries and venules, were performed on the same
cryosection. When required for visibility, images shown of the same antigen on different vessels
were enhanced identically with identical linear adjustments of contrast applied uniformly to
every pixel. Quantitative pairwise comparisons of at least ten specimens of each vessel type
were performed. Umbilical artery and vein were identified by their arrangement within the cord. Coronary artery and vein were distinguished by the distinct thickness of the tunica media. Dermal capillaries were identified as small UEA-I positive vessels within the dermal papillae. Dermal arterioles and venules of the superficial vascular plexus were identified as UEA-I bright paired vessels deep to the papillae and distinguished from each other by the larger size of the venule. Lymphatic vessels stain poorly with UEA-1 and were excluded from the analysis. In order to compare relative claudin-5 and VE-cadherin expression in umbilical cord artery vs. vein and of coronary artery vs. vein, epifluorescence microscope images were analyzed with Volicity 6.1.1 software cellular imaging tools (Perkin Elmer), tracing each vessel outline to create a region of interest (ROI) defining a contiguous region of immunofluorescence generated by immunostaining at serial dilutions of primary antibody. Mean pixel intensity levels were automatically generated from replicate samples at each antibody concentration that were corrected by subtracting mean intensity levels of background immunofluorescence gathered from ROIs drawn in surrounding tissue where no VE-cadherin or claudin-5 expression is present. For skin microvessels, thresholds for positive immunostaining were determined by visually inspecting vascular structures positive for UEA-1 and comparing immunofluorescence intensity of isotype matched negative control antibodies to that from titrations of antibodies specific to claudin-5, VE-cadherin or ZO-1. The threshold for positive immunostaining was reached when a side-by-side comparison of each specific antibody to isotype control showed a difference obvious to three observers. This threshold was then used as reference for comparisons at each concentration of antibody titration. The data are then analyzed as the percentage of vessels of each type that stain positively for the antigen of interest at each dilution of antibody. By means
of confocal fluorescence microscopy, observations made by epifluorescence microscopy were confirmed and are displayed at informative antibody dilutions in Supplemental Fig II.

**Endothelial cell cultures**

HDMECs were isolated from the superficial vascular plexus of normal adult human skin obtained as above from anonymized donors. The superficial most 0.5 to 0.7 mm were harvested by dermatome slicing, followed by fine mincing and enzymatic digestion (Dispase, 50 U/ml; BD Biosciences) for 30 min at 37°C as described previously. Liberated cells were cultured on 5 mg/ml human plasma fibronectin-coated tissue culture plastic (BD Biosciences) in EGM2-MV growth medium (Lonza) that was replaced at 48 h intervals. After five days in primary culture, the cell populations were washed to remove non-adherent cells and debris, following which the substrate-adherent cells were resuspended using trypsin and then immunoselected using anti-CD-31-biotin followed by streptavidin-magnetic beads (Miltenyi Biotec). Replated HDMECs were serially passaged on 0.1% gelatin-coated plastic in the same medium and used between passage 4-7. HUVECs from two or three umbilical cords were released by collagenase digestion, pooled and cultured in M199 medium/20% FBS supplemented with 2.3 mM L-glutamine, penicillin/streptomycin (200 U/ml, Invitrogen), endothelial cell growth supplement (0.05 mg/ml, Calbiochem) and heparin (0.1 mg/ml, Sigma) as previously described. HUVEC cultures established in this manner were then weaned gradually into EGM2-MV medium and used between passage 4-6. The medium was generally replaced with fresh medium 48 h before specific measurements were made. In this study EC monolayers were assayed starting on day 0 post-visual confluence, which we define as a little or no visible cell culture plastic substrate and
when ECs have attained physical contact with neighboring EC. At post-visual confluence HDMEC and HUVEC monolayers show similar magnitudes of decrease in mean cell surface area and increase in cell numbers (unpublished data). At post-visual confluence both HDMEC and HUVEC monolayers show up to a 304% increase in total cell number and a 58% decrease in cross-sectional area, but these changes were equivalent in both cell types.

**DNA constructs and transductions**

An IL2R-VE retroviral construct was assembled from cDNA of the IL2R-VE-cadherin fusion protein, consisting of the human IL2Rα (CD25) extracellular and transmembrane domains fused to the human VE-cadherin cytoplasmic domain in a pCMV plasmid kindly provided by Dr. Andrew Kowalczyk (Emory University).³ Zero Blunt TOPO PCR cloning was performed to transfer the coding sequence for IL2R-VE-cadherin into a pCR-BluntII-TOPO vector (Invitrogen) with sense primer 5’-ACCATGGATTACATCATACCTGCTG-3’ and antisense primer 5’-CTAATACAGCAGCTCCTCCC-3’ and then ligated into the EcoR1 sites of the retroviral vector pLZRS.CMV.⁴ A human claudin-5 retroviral construct was assembled from human claudin-5 cDNA (clone ID 5242567 obtained from Open Biosystems) and sub-cloned into the HindIII and NotI sites of the retroviral vector pLZRS.CMV after which a Kozak consensus sequence was inserted upstream of the ATG start site (QuikChange II XL Site-Directed Mutagenesis Kit, Stratagene). The identical retroviral vector, but without any cDNA insert served as a negative control for each pLZRS.CMV construct. The retroviral EGFP-claudin-5 construct was assembled from cDNA of an N-terminal EGFP-sequence fused to the full length human wild-type cDNA sequence of claudin-5 within the pEGFP-C1-vector (Clontech).⁵ The cDNA encoding this fusion
protein was TOPO cloned into pCR-BluntII-TOPO vector with sense primer 5’-CGCCACCATGGTGAGCAAG-3’ and antisense primer 5’ TCAGTTATCTAGATCCGTTGA-3’.

Subsequently, the EGFP-claudin-5 insert was ligated into the BamHI and SalI sites of the retroviral vector pBMN.neo. The identical retroviral vector but with an insert consisting of EGFP, served as a negative control. Insert sequences from these three overexpression constructs were verified as correct by DNA sequencing. Human GIPZ lentiviral shRNAmir constructs used for lentivirus knockdown obtained as glycerol stabs from Open Biosystems were: for human claudin-5, clones V2LHS_171415 (5’-TTTACTAAGCAGATTCTTAGCC-3’) and V2LHS_171412 (5’-TATCCAACGCCTCGCAGGC-3’); for human occludin, clone V2LHS_152425, (3’ UTR: 5’-TTAATTTCCTAAACAACCTTGCC-3’). A non-silencing GIPZ lentiviral shRNAmir verified to contain no homology to known mammalian genes (Catalog number RHS4346) or the pGIPZ empty vector (Catalog No. RHS4973) served as controls. Retroviral supernatants in DMEM medium supplemented with 10% FBS produced from transected packaging cell lines (Phoenix cells for PLZRS constructs; GP-293 cells for pBMN constructs) were used for transducing ECs without drug selection (pLZRS vectors) or with (pBMN.neo vectors) 1 mg/ml G418 (Invitrogen) selection. Lentivirus supernatants produced by co-transfecting TLA-HEK-293T cells (OpenBiosystems) with individual pGIPZ shRNA plasmids, the psPAX2 helper plasmid (kindly provided by Dr. Alfred Bothwell, Yale University) and the pLP/VSV-G envelope plasmid (Invitrogen) were used to transduce both EC types that were subsequently selected in puromycin (1 mg/ml). All EC lines were assessed for transduction efficiency (by FACS analysis) and for overexpression or knockdown (by Western analysis) prior to experimental use.
FACS analysis and immunoblotting

For FACS analyses of junctional molecule expression, ECs cultured to day 3 post-visual confluence were resuspended using trypsin, quenched in 10% FBS-containing medium, rinsed in 1% BSA in PBS and incubated for 30 min at 4°C in the same buffer containing fluor-conjugated primary antibody or isotype-matched control. When antibody detection of intracellular epitopes required permeabilization, EC suspensions were fixed in 4% paraformaldehyde/PBS for 15 min at 4°C, rinsed twice and incubated in the presence of antibodies in 1% FBS/0.1% saponin/PBS. After three more rinses in 1%BSA/PBS (with BSA replaced with FBS and saponin for permeabilized ECs), FACS analyses were performed on a Becton-Dickenson LSRII equipped with blue (488 nm) and red (633 nm) lasers. For immunoblot analyses, ECs cultured in C6 plastic wells (BD Biosciences) to the timepoints indicated were scrape-harvested on ice into 500 ml Laemmli sample buffer (62.5% mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue) supplemented with 20 mM dithiothreitol (Sigma), 5 mM EDTA (Invitrogen), complete protease inhibitor and PefaBloc SC (both from Roche), flash frozen on dry ice, boiled for 10 min, syringe-sheared (30 gauge) three times to dissipate DNA, then boiled 3 min. Supernatants fractionated on SDS-PAGE gels were transferred onto PVDF filters (Millipore) at 120 volts at 4°C, blocked in 5% non-fat dry milk in 50 mM Tris-base, 150 mM NaCl, pH 7.4, 0.05% Tween-20, then incubated with primary antibody overnight with rocking at 4°C in blocking buffer. After incubation with species-specific horse radish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) detection of bound antibodies was performed with enhanced chemiluminescence (Amersham). Results were quantified by densitometry with a Gel-Doc XR using Quantity One version 4.6.5 Software (Bio-Rad).
Confocal and epifluorescence immunomicroscopic analyses of cells

Immunomicroscopic analyses were performed on ECs seeded onto fibronectin-coated glass cover slips (Fischer Scientific) at day 3 post-visual confluence unless indicated. ECs were rinsed in Dulbecco’s PBS (Sigma) with Ca++ and Mg++ added as 1.33 g/L CaCl₂·2 H₂O and 1 g/L MgCl₂·6H₂O, fixed in either 4% paraformaldehyde for 10 min followed by (if permeabilizing) 0.2% Triton-X-100 for 10 min at RT, or (for detecting claudin-5) in ice-cold 95% EtOH for 30 min followed by washing/re-hydration in PBS with Ca++ and Mg++. After blocking in 5% donkey serum (Jackson ImmunoResearch)/1%BSA/PBS with Ca++ and Mg++ (if permeabilized) 0.1% saponin for 1 h at RT (blocking buffer), EC were incubated in primary antibodies in the same blocking buffer overnight at 4°C. After washing in PBS with Ca++ and Mg++ plus 1% BSA (wash buffer) signals were detected by incubating 1 hour at RT with Alexa Fluor-conjugated affinity-purified donkey anti-mouse or anti-goat secondary antibodies (Invitrogen) in blocking buffer. After washing, ECs were coverslipped and mounted in ProLong® Gold antifade reagent with DAPI (Invitrogen) for detecting nuclei. In Figures 4, 6 and Supplemental Figures I, II, VIII and IX immunofluorescence images were obtained with a spectral confocal microscope (Leica TCS SP5, Leica Microsystems) equipped with acousto-optical filters, an acousto-optical beam splitter and a Leica SP prism spectrophotometer detection system (using a HCX PL APO lambda blue 63X magnification, oil, numerical aperture 1.40 lens) and in other figures of ECs with an epifluorescence microscope (Axiovert 200M Carl Zeiss using a Plan-APochromat 63X magnification, oil, numerical aperture 1.4 lens or a LD Plan-Neofluar 40X magnification, air, numerical aperture 0.6 lens).
Transmission electron microscopy

ECs, isolated and propagated as described above, were seeded onto fibronectin-coated high-density 0.4 mm pore size 6 well format cell culture inserts (BD Biosciences) and on day 3 post-visual confluence were washed in PBS with Ca\(^{++}\) and Mg\(^{++}\), processed in fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M sodium cacodylate (Caco) buffer pH 7.4 from Electron Microscopy Services) for 1 h at RT, then stored in Caco buffer at 4°C until processing. Specimens were then post-fixed in osmium tetroxide for 1 hour at RT, followed by one rinse in Caco buffer, then 3 rinses in 50 mM sodium maleate buffer pH 5.2. Samples were then immersed in 5% uranyl acetate in the same maleate buffer for 1 hour in the dark, rinsed 3 times in distilled water and then dehydrated through a graded ethanol series from 50%-100%. The 100% ethanol was exchanged with 3 rinses of propylene oxide and the specimens were then embedded in Epon. The blocks were baked at 60°C overnight. Apical to basolateral thin sections were cut with a Leica Ultra trim microtome and sections were stained for contrast with 2% uranyl acetate and lead citrate and viewed in a Tecnai 12 BioTwin transmission EM. TJ ultrastructures were identified by transmission EM imaging as sites where membranes converge as “kissing points” or establish electron dense material indicative of intracellular cytoplasmic plaque proteins.\(^7,8\)

Transendothelial flux and TEER measurements

For transendothelial flux measurements, ECs were seeded onto fibronectin-coated 0.4-mm pore polyethylene terephthalate 24-well size cell culture inserts (BD Falcon). FITC-dextrans (1 mg/ml, either 3 kD, catalog No. D3306 or 70 kD, catalog No. D1822 from Invitrogen) suspended
in EBM basal medium (base medium for EGM-2MV, Lonza) were added into the apical chamber. After incubation at 37° C or at 4° C for the times indicated, medium from the bottom chamber was collected and the concentration of FITC dextran measured using an automated fluorescence plate reader, Bio-Rad Model 680, and is reported minus basal medium background. FACS analysis, used to verify that internalization of 3 kDa FITC-dextran probes by both EC types was inhibited at 4° C, indicated that acid wash resistant signal intensities resulting from uptake of 3 kDa FITC-dextran were 84% and 88% less for HDMEC and HUVEC, respectively for 6 hour incubations at 4° C compared to parallel incubations at 37° C. EC barrier function was also assessed by ECIS (Applied BioPhysics). ECs were cultured on fibronectin-coated 8W10E + gold electrode 8-chamber slides and measurements were taken at 24 h intervals at 4,000 Hertz to determine TEER.

**Calcium chelation assay**

Images documenting gap formation or VE-cadherin displacement were acquired at 5 to 10 minutes after adding EGTA.

**Statistics**

Significance of differences between two groups was tested by an unpaired t-test or among more than two groups, by one-way ANOVA followed by the Dunnet’s Multiple Comparison Test or two-way ANOVA followed by the Bonferroni post-test correction. P-values < 0.05 were accepted as significant. All results were computed using Prism version 4.0c and are presented as means +/- SEM (unless indicated as SD).
Supplemental Figure Legends

**Supplemental Figure I.** Confocal maximum intensity projections of human umbilical vessels and human cardiac vessels for claudin-5 and VE-cadherin expression *in situ*. (A and B) Human umbilical artery and vein stained with FITC-Ulex Europeus Agglutinin I (UEA-I) and assessed for expression levels of (A) claudin-5 and (B) VE-cadherin by immunostaining with rabbit anti-claudin-5 or rabbit anti-VE-cadherin primary antibodies at 1:5000 dilutions. Representative of two umbilical cord specimens analyzed with similar results. Scale bar = 100 µm. (C and D) Human coronary artery and vein stained with DAPI, FITC-Ulex Europeus Agglutinin I (UEA-I) and assessed for expression levels of (A) claudin-5 and (B) VE-cadherin by immunostaining with rabbit anti-claudin-5 or rabbit anti-VE-cadherin primary antibodies at 1:5000 dilutions. Representative of coronary artery and vein specimens from two different donors analyzed with similar results. Scale bars = 75 µm.

**Supplemental Figure II.** Confocal maximum intensity projections of human dermal microvascular segments identified by UEA-I staining are immunostained for claudin-5 and VE-cadherin expression *in situ*. Immunostaining for (A) Claudin-5, (B) VE-cadherin and (C) ZO-1 with rabbit anti-claudin-5, rabbit anti-VE-cadherin or mouse anti-ZO-1 primary antibodies at 1:2000, 1:5000 or 1:1000 dilutions, respectively. Representative of two skin specimens analyzed with similar results. Scale bars = 50 µm. Capillaries (cap) yellow arrows; arterioles and venules (a/v) white arrowheads and white arrows, respectively; ep”, epidermis.
Supplemental Figure III. Assessment of whether the composition of pre-formed matrix substrate underlies differences in barriers formed by HDMEC and HUVEC monolayers. TEERs of HDMEC and HUVEC monolayers plated onto human fibronectin (5 µg/ml) or collagen IV (20 µg/ml). Statistical significance assessed by two-way ANOVA with Bonferroni post-test correction was not significant for fibronectin vs. collagen in either HDMEC or HUVEC. Bars represent SEM across different electrodes (n = 8, 8, 8, 8). Representative of two experiments comparing HDMEC on different matrices with similar results.

Supplemental Figure IV. Transendothelial flux of 3 kD FITC-dextran over 6 h performed at 37° C and 4° C. Differences in the level of flux between HDMEC and HUVEC monolayers is presented as means +/-SEM and error bars represent variance among different transwells (n = 6,6,6,6). Statistical significance was assessed by unpaired two-tailed t-test with P values as indicated.

Supplemental Figure V. (A) Immunoblotting of ZO-1 from replicate HDMEC and HUVEC monolayers harvested on day 0, 1 and 3 post-visual confluence. (B) Densitometric analyses from three experiments comparing HDMEC and HUVEC expression of ZO-1 normalized to that of β-actin. Statistical significance relative to expression on Day 0 post-confluence assessed by two-way ANOVA with Bonferroni post-test correction was not significant for HDMEC or HUVEC.

Supplemental Figure VI. Calcium chelation in HDMEC and HUVEC cultures. (A) ECIS analysis of TEER on day 3 post-visual confluence of HDMEC or HUVEC monolayers conducted 5 minutes after exchange of basal medium with fresh medium containing 1% BSA (control), or the same
medium supplemented with EGTA, with EGTA plus excess Ca++, or EGTA plus excess Mg++. Differences vs. medium control are statistically significant for HUVEC (*P < 0.0001 by one-way ANOVA, Dunnet’s Multiple Comparison Test) but not for HDMEC monolayers. Representative of two independent experiments analyzed with similar results. (B) Epifluorescence micrographs of HDMEC or HUVEC monolayers at day 3 post-visual confluence immunostained with anti-CD31/PECAM-1 (in permeabilized cells) to highlight gap formation 5 min following exchange of medium for fresh medium supplemented with 1% BSA with or without EGTA. Gaps formed between HUVEC but not HDMEC as indicated by arrows. Scale bar = 20 µm. (C) Epifluorescence micrographs of HDMEC and HUVEC monolayers +/- calcium chelation with EGTA immunostained with goat anti-VE-cadherin following permeabilization. Note that after EGTA treatment the majority of VE-cadherin is displaced away from HUVEC junctions but is retained at HDMEC junctions. Scale bar = 30 µm.

Supplemental Figure VII. Junctional organization of EGFP-claudin-5 overexpressed in ECs. (A) Histograms of FACS analyses of G418-selected HUVECs transduced with either pBMN.neo.EGFP control vector (solid histogram, left) or pBMN.neo.EGFP-claudin-5 (solid histogram, right); open histograms represent background fluorescence by non-transduced HUVEC cultures (negative control). (B) Immunoblot analysis of protein lysates from HUVEC EGFP (lane 1) or EGFP-claudin-5 (lane 2; EGFP-CL5) transductants. Numbers indicate densitometric analyses of the immunoblot data normalized to expression of β-actin. Note that VE-cadherin levels were unaffected by overexpressing EGFP-claudin-5. (C-F) Fluorescence micrographs of unfixed HUVEC- (C and D) and HDMEC- (E and F) transductants. HUVEC EGFP-claudin-5 transductants
are shown on day 0 (C) and on day 5 (D) post-visual confluence. HDMEC EGFP-claudin-5 transductants are shown on day 0 (E) and day 5 (F). Note that EGFP-claudin-5 protein in HUVEC transductants remained saw-tooth and fragmented at the junctions through day 5 (arrow in panel D) but by day 5 post-confluence EGFP-claudin-5 protein has assembled into a focused, continuous pattern at some cell junctions of HDMEC transductants (arrow in panel F). Scale bar = 15 µm. One of two experiments with similar results.

**Supplemental Figure VIII.** TEER measurements and EGTA-induced gap formation response of HUVEC overexpressing untagged claudin-5. (A) Immunoblot analysis of HUVECs transduced with empty vector pLZRS (lane 1, negative control) or with an otherwise identical retroviral construct encoding Cld5 (lane 2; pLZRS-CL5). Numbers indicate densitometric analyses of the immunoblot data normalized to expression of β-actin. (B) Confocal fluorescence micrographs of pLZRS control-transduced HUVECs (top) and pLZRS.Cld5-transduced HUVECs (bottom) immunostained with rabbit anti-Cld5 (red) and counterstained with DAPI (blue). Arrows highlight increased immunostaining in the Cld5 transductants. (C) TEER measurements of Cld5-transduced and empty vector control-transduced HUVEC monolayers presented as mean +/- SEM. Bars represent SD among different electrodes (n = 8, 8). Small differences measured vs. controls were statistically significant on day 0, 2, 3 and 4 post-visual confluence with P values < 0.01, <0.05, <0.001 and <0.001, respectively by two-way ANOVA, Bonferroni post-test correction, but TEER remained below 2000 ohms. (D) Confocal fluorescence micrographs of empty vector- vs. Cld5-transduced HUVEC monolayers 10 min following replacement of the medium with basal medium containing BSA (left) or the same medium containing EGTA (right).
Arrows highlight gap formation that was inhibited by Cld5 overexpression as revealed by immunostaining with anti-CD31/PECAM-1 antibody. Scale bar = 15 μm.

**Supplemental Figure IX. shRNA knockdown of occludin in HUVEC cultures.** (A) Immunoblot analysis of HUVEC transduced with lentivirus shRNA to Cld5 (CL5 KD) or to occludin (Occ KD) vs. non-silencing lentivirus vector control (Control). (B) Confocal immunofluorescence microscopy of junctional expression in HUVEC. Scale bar = 25 μm. (C) Effect of occludin shRNA knockdown on HUVEC TEER levels recorded daily from visual confluence. Bars represent SD among different electrodes (n = 8, 8). Representative of three independent experiments analyzed with similar results.
Supplemental References


5. Ruffer C, Gerke V. The C-terminal cytoplasmic tail of Claudins 1 and 5 but not its PDZ-binding motif is required for apical localization at epithelial and endothelial tight junctions. *Eur J Cell Biol.* 2004;83:135-144


Supplemental Figure I

A.

Umbilical Artery

UEA-I

Claudin-5

Umbilical Vein

B.

Umbilical Artery

UEA-I

VE-cadherin

Umbilical Vein
Supplemental Figure I (continued)

C.

Coronary Artery

Coronary Vein

D.

Coronary Artery

Coronary Vein
Supplemental Figure III

![Graph showing TEER (ohms) against Day post-Visual Confluence for different cell types and substrates.](image)

- **ECIS**
- **Axes:**
  - Y-axis: TEER (ohms)
  - X-axis: Day post-Visual Confluence

- **Legend:**
  - HDMEC Fibronectin
  - HDMEC Collagen
  - HUVEC Fibronectin
  - HUVEC Collagen

- **Data Points:**
  - Points are plotted at various days post-visual confluence, showing trends in TEER for each condition.
**Supplemental Figure IV**

- HDMEC at 37 deg. C
- HDMEC at 4 deg. C
- HUVEC at 37 deg. C
- HUVEC at 4 deg. C

![Graph showing Dextran-FITC concentration (ug/ml in lower chamber) for HDMEC and HUVEC at different temperatures.](image)

- *P < 0.0001
- **P < 0.0001
- N.S.
Supplemental Figure V

Day Post-Confluent:

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<tr>
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Antibody
ZO-1

β-actin

M.W.
220 kDa
42 kDa

Fold-Change vs. Day Zero

Day post-Visual Confluence
Supplemental Figure VII

A. 

**EGFP-Control**

EGFP positive subset 92.3%

**EGFP-Claudin-5**

EGFP positive subset 59.7%

B. 

**HUVEC**

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<tr>
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**VE-cadherin**

Densitometry: 1.6 1.7

**β-actin**


C. 

**EGFP Fluorescence**

HUVEC HDMEC

Day 0

D. 

Day 5

E. 

Day 0

F. 

Day 5
Supplemental Figure VIII

A. Transduced: HUVEC
   Antibody          M.W.  
   Claudin-5         23 kDa  
   Densitometry: 0.61 1.13
   VE-cadherin      
   Densitometry: 1.23 1.25
   β-actin          
   42 kDa

B. Transduced: HUVEC: anti-Claudin-5
   Vector Control
   Untagged Cld5

C. TEER (ohms)
   Day post-Visual Confluence
   Untagged CL5
   Vector Control

D. Transduced: HUVEC: anti-CD31
   Vector Control
   Untagged Cld5
   Medium
   +EGTA
Supplemental Figure IX

A.  

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B.  

Post-confluence:  

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C.  

[Graph showing TEER (ohms) over Day post-Visual Confluence for Vector Control and ShRNA Occludin]