Dual Roles of Tight Junction Associated Protein, Zonula Occludens-1, in Sphingosine-1-phosphate Mediated Endothelial Chemotaxis and Barrier Integrity

Jen-Fu Lee¹, Qun Zeng¹, Harunobu Ozaki², Lichun Wang¹,³, Arthur R. Hand⁴, Timothy Hla⁵, Eugenia Wang¹,⁶, Menq-Jer Lee¹,³,⁷

¹: Gheens Center on Aging, University of Louisville Health Sciences Center.  
²: Dept of Cardiology, Keihanna hospital, 1-2-1 Fujisakahigashi-cho, Hirakata, Osaka, Japan 573-0153.  
³: Department of Microbiology and Immunology, University of Louisville Health Sciences Center.  
⁴: Department of Pediatric Dentistry, University of Connecticut Health Center, Farmington, CT, USA.  
⁵: Center for Vascular Biology, Department of Physiology, University of Connecticut Health Center, Farmington, CT, USA.  
⁶: Department of Biochemistry and Molecular Biology, University of Louisville Health Science Center.  
⁷: Corresponding Author: Menq-Jer Lee, Ph.D.  
University of Louisville Health Sciences Center  
580 S. Preston St. Louisville, KY 40202, USA.  
Tel: 502-852-7074  
Fax: 502-852-2660  
Email: menqjer.lee@louisville.edu

Running title: ZO-1 in S1P-regulated endothelial chemotaxis and barrier integrity
In this report, sphingosine-1-phosphate (S1P), a serum-borne bioactive lipid, is shown to activate tight-junction associated protein Zonula Occludens-1 (ZO-1), which in turn plays a critical role in regulating endothelial chemotaxis and barrier integrity. After S1P stimulation, ZO-1 was redistributed to the lamellipodia and cell-cell junctions via the S1P1/Gi/Akt/Rac pathway. Similarly, both endothelial barrier integrity and cell motility were significantly enhanced in S1P-treated cells through the Gi/Akt/Rac pathway. Importantly, S1P-enhanced barrier integrity and cell migration were abrogated in ZO-1 knockdown cells, indicating ZO-1 is functionally indispensable for these processes. To investigate the underlying mechanisms, we demonstrated that cortactin plays a critical role in S1P-induced ZO-1 redistribution to the lamellipodia. In addition, S1P significantly induced the formation of endothelial tight junctions. ZO-1 and α-catenin polypeptides were colocalized in S1P-induced junctional structures; whereas, cortactin was not observed in these regions. Together, these results suggest that S1P induces the formation of two distinct ZO-1 complexes to regulate two different endothelial functions: ZO-1/cortactin complexes to regulate chemotactic response and ZO-1/α-catenin complexes to regulate endothelial barrier integrity. The concerted operation of these two ZO-1 complexes may coordinate two important S1P-mediated functions, i.e. migration and barrier integrity, in vascular endothelial cells.

Endothelial barrier integrity is an important physiological function of the endothelium in vivo. Dysregulated barrier integrity is implicated in a variety of pathological conditions, such as stroke, inflammation, various immune responses, etc (1). To elucidate the function and regulation of endothelial barrier integrity, cultured brain microvascular endothelial cells have been widely employed as an in vitro model system to study the blood–brain barrier (BBB) (2). Evidence from these studies indicates that BBB plays a critical role in regulating the homeostatic environment of the brain and the transportation of plasma constituents into brain. Furthermore, it has been shown that severely impaired blood-brain barrier integrity is attributed to the pathological states of various neurological disorders, such as multiple sclerosis (3, 4), Alzheimer’s disease (5, 6), and human immunodeficiency virus-1–associated encephalitis or dementia (7, 8).

Sphingosine-1-phosphate (S1P), a serum-borne bioactive lipid mediator secreted by activated platelets (9), enhances barrier formation in cultured pulmonary endothelial cells (ECs) (10). However, the molecular details for the formation and maintenance of endothelial barrier integrity are poorly understood. It was recently reported that the association of cortactin, an F-actin cross-linking polypeptide, and myosin light chain kinase is crucial in S1P-enhanced endothelial barrier integrity (11). Furthermore, it is well documented that tight junctions are important in regulating BBB formation (12, 13). In addition, S1P greatly enhances VE-cadherin based adherens junctions in endothelial cells (14, 15). Also, platelet–endothelial cell adhesion molecule-1 (PECAM), E-selectin, and intercellular cell adhesion molecules (ICAM) are regulated in endothelial cells with S1P treatment (16). Together, S1P-mediated endothelial barrier function may be the concerted activation of these molecules required for junctional structures and cell-cell interaction.

Here we demonstrate that S1P treatment not only significantly increases endothelial barrier integrity, but also greatly enhances cell migration into electrically
wounded areas, as determined by Electrical Cell-substrate Impedance Sensing technology (ECIS) (17). In addition, both endothelial barrier integrity and chemotactic response were found to be controlled by the same signaling cascade, i.e. S1P1/ Akt/ Rac pathway. Thus, it suggests that a common modulator may be present to regulate both responses. In this report, evidence is shown that Zonula Occludens-1 (ZO-1), a tight junction associated protein, is functionally critical in regulating S1P-mediated endothelial barrier integrity and chemotactic response. Data from this study also suggests that S1P induces the formation of distinct ZO-1 functional complexes, which in turn control the distinct physiological processes of the endothelium.

**Experimental Procedures**

**Materials, cell culture, and adenoviral transduction:** Sphingosine-1-phosphate was obtained from Biomol. Pertussis toxin and Ly294002 were from Calbiochem. Other reagents, unless specified, were from Sigma. Human Umbilical Vein Endothelial Cells (HUVECs) and Chinese Hamster Ovary (CHO-K1) cells stably expressing the S1P1 receptor were cultured essentially as described (18). Human brain microvascular endothelial cells (HBMECs, gift of Dr. Kwang Sik Kim, Johns Hopkins University) were cultured essentially as HUVECs. For adenoviral transduction, cells were infected with adenoviral particles carrying various cDNA constructs for 12 hr prior to stimulation with S1P as previously described (18, 19).

**siRNA mediated gene silencing**: To specifically knockdown the endothelial ZO-1 polypeptide, two pairs of sense and anti-sense hairpin oligonucleotides flanked with BamH1 and EcoRI restriction sites at 5’-and 3’-end, respectively, were designed according to the GenScript siRNA sequence design tool (siRNA target finder, https://www.genscript.com/ssl-bin/app/rnai). The oligonucleotides sequences for the construct #1: (sense): 5’-gatcc GTGAG CAATA TTCAC GCAGT TATTCT AAGAG ATAAC TCGGT GAATA TTGCT CACTT TTTTg-3’, (antisense): 5’-aattc AAAAA AGTGA GCAAT ATTCA CGCAG TTATC TCTTG AATAA CTGCG TACAG-3’; and construct #2: (sense) 5’-gatcc GTTAT TCGCC TGATG ACAAT ATCTC AAGAG ATGAT TGTAT GCAGC GAAT AACTT TTTTg-3’, (antisense): 5’-aattc AAAAA AGTGA TCCGC TGGCA TACAA TAATC TCTTG AATTA TTGTA TGCAG GCGAA TAAAg-3’. The sense and anti-sense oligonucleotides were annealed and cloned into the Lentiviral pFIV-H1 vector (System Bioscience). Lentiviral transducing particles were produced by packaging in the 293T/17 cell line following the manufacturer’s instructions. Lentiviral particles carrying the siRNA oligonucleotide for luciferase were used as a control in this study. HUVECs (6 x 10^5 cells) were infected with Lentiviral particles (~ 0.1 m.o.i.) in 60 mm dishes for 24 hr in the presence of polybrene (2 µg/ml), washed and replaced with fresh medium for additional 24 hr. Subsequently, cells were split (1:3 split ratio), and stably transduced HUVECs were isolated with Puromycin (1 µg/ml) selection. In addition, two pairs of oligonucleotides were used to knockdown S1P1 receptor, the construct #23: (sense) 5’ – gatcc GCAC GTATC CTTCT CTGCT TCAAG AGAGC AGAAG ATAGT ATAGT GTTGT – 3’, (antisense) 5’ – aattc AAAAA AGCAC TATAT CATTCT CTTCT TGAAG CAGAA GAGGA TATAG TGCG – 3’; the construct #24: (sense) 5’ – gatcc GTCTA CTCCT TGGTC AGGAC TTTCA AACTT TTTTg – 3’, (antisense) 5’ – aattc AAAAA ATCTA CTCCT TGGTC AGGAC TTTCA GAGAG TCTCT TGAAG CAGAA GAGGA TATAG TGCG – 3’.
**Immunofluorescence analysis:** Recently confluent HUVECs (3 x 10^5 cells in 35 mm glass bottom dishes, MatTek) were serum-starved in plain M199 medium for 2 hr, followed by stimulation with S1P (500 nM, 30 min). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton-X100, and immunostained with the indicated primary antibodies overnight. Following incubation with Alexa488- or Alexa594-conjugated secondary antibodies (Molecular Probes), the stained images were visualized and photographed with the Axiovert 200M epi-fluorescence microscope (Carl Zeiss).

**Western blot analysis:** Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the membranes were blotted with 1 µg/ml of ZO-1 (Zymed) or cortactin antibody (4F11, Upstate). Subsequently, the nitrocellulose membranes were incubated with their corresponding HRP-conjugated secondary antibody (Pierce) and visualized using the ECL method (Amersham).

**Phosphothioate oligonucleotide transfection:** The following 15-mer phosphothioate oligonucleotide (PTO) was synthesized to block the expression of cortactin polypeptide: antisense cortactin, 5'-AGCGTGCCCTGCTGA-3'. The sense PTO, 5'-TCAGCAGGCCACGCT-3', was used as a control. For PTO transfection, HBMECs (2 x 10^6 in 100 mm dish) were co-transfected with PTO (100 nM) and cDNA vector of green fluorescent protein (GFP, 8 µg) (15) for overnight by lipofectamine reagent. Subsequently, cells were serum-starved in medium M199 supplemented with 0.5% FBS for 24 hrs. After stimulating without or with S1P (500 nM) for 30 min, cells were subjected to immunoblot or immunostaining as described above.

**Transmission electron microscopy:** HUVECs (1 x 10^5 cells) were plated in tissue culture flasks (12.5 cm^2, Falcon). Three days later, cells were starved in plain M199 for 2 hrs, followed by stimulating without or with S1P (500 nM, 30 min). Cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide-0.8% potassium ferricyanide in cacodylate buffer, treated with 0.5% aqueous uranyl acetate and embedded in epoxy resin. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and observed in a Philips CM10 electron microscope at 60 kV.

**Electrical cell-substrate impedance sensing (ECIS) assay:** ECIS™ Model 1600R (Applied BioPhysics) was used to measure the transendothelial electrical resistance (TEER) in confluent or electrically wounded endothelial cells to study the S1P-regulated barrier integrity and migratory response, respectively (17). Briefly, to study the endothelial integrity function, 200 µl of cell suspension (5 x 10^5 cells per ml) were seeded to each well of either a 8W1E or 8W10 ECIS array (one electrode or 10 electrodes per well, respectively), which was pre-equilibrated with 200 µl of medium at 37°C for 30 min. Two-three days later, endothelial cells were serum-starved in plain M199 medium for 2 hours at 37°C. After stimulation with S1P, the endothelial integrity function was measured in real-time as described (17). For migratory response, the cell-covered electrode was connected to an AC source. By applying 5 volts for 30 second, a relatively high current was delivered to the cell-covered electrode and killed the cells on the electrode (250 µm diameter) (17). Thereafter, endothelial migratory responses were determined in real-time by measuring the recovery of electrical impedance, an indicator of the surrounding viable endothelial cells migrating into the wounded area, in the presence or absence of S1P treatment.

**Migration assay:** Endothelial chemotaxis was measured by using the transwell migration assay (8 µm pore size, Costar) as described
Briefly, polycarbonate membranes were coated with human Fibronectin (1 µg/ml) for overnight at 4°C. Following serum starvation, HUVECs were trypsinized, counted, and resuspended in M199 media with 0.5% fatty-acid free BSA (Sigma). Cells (7.5 x 10⁴ cells in 100 µl) were placed in the upper chamber and media (600 µl) containing various concentrations of S1P were added to the lower chamber. Endothelial cells were allowed to migrate for 4 h at 37 °C in a humidified chamber with 5% CO₂. Subsequently, the nonmigrated cells on the upper side of the filter were removed with a cotton swab. The filters were fixed with 4% formaldehyde and stained with 0.1% crystal violet solution. Following eluting the stained dye with 10% acetic acid, cell chemotaxis was quantitated with BMG FluoStar Galaxy microplate reader (BMG Labtechnologies) at O.D. 590 nm. Statistic analysis: t-test was used for statistic analysis. p-value < 0.001 was considered as statistically significant.

Results

To study the dynamic behavior of endothelial cells in culture, the Electrical Cell-substrate Impedance Sensing technology (ECIS) was used to measure the S1P-regulated transendothelial electric resistance (TEER) in real time (17). In this technology, as endothelial cells attach and spread on the small gold film electrode of the ECIS array, cellular membranes restrict the electrical current, forcing it to flow beneath and between the cells, resulting in a dramatic increase in electrical impedance (Fig. 1a). Furthermore, the endothelial cells attached to the circular microelectrode (250 µm diameter) can be selectively killed by applying a high-voltage electrical current (5 volts for 30 seconds, arrowhead in Fig. 1a). The efficiency of this electrical current to completely kill endothelial cells attached to the microelectrode was shown by an abrupt drop of electrical impedance (Fig. 1a) and was verified by microscopic observation (Panels I & II, Fig. 1b). Subsequently, the viable cells surrounding the wounded microelectrode were allowed to migrate into the electrically wounded area, and reestablished the cell-cell interaction over the electrode (Panels III & IV, Fig. 1b). The kinetics for this sequence of events can be monitored in real-time by the increase of electrical impedance (Fig. 1a).

We utilized the ECIS technique to monitor the spatial and temporal behavior of S1P-regulated migration in repairing the endothelial injury. Endothelial cells attached to the circular microelectrodes were killed by elevating electrical current and the surrounding viable cells were allowed to migrate into the wounded area in the presence or absence of S1P. As shown in Fig. 2a, S1P treatment significantly increased the TEER in the electrically injured endothelial monolayers. Microscopic examination showed that the raised TEER correlated well with the numbers of cells migrated into the wounded area (Fig. 2b). Moreover, this S1P effect on cell motility was markedly diminished in S1P1 knockdown ECs (si-S1P1) (Fig. 2c), indicating S1P1 receptor play a critical role in this process. The reduced but detectable S1P-responsiveness in si-S1P1 cells may be due to the residual S1P1 receptor in knockdown cells (Fig. 2e). It was shown that S1P is able to activate Akt and Rac GTPase in endothelial cells (18), and S1P-regulated cytoskeletal remodeling and chemotactic response are dependent on Akt and Rac activities (14, 18). As shown in Fig. 2d, the S1P-induced cell migration in ECIS wound-healing assay required Akt and Rac activities, because transduction with adenoviral particles carrying dominant-negative -Akt and -Rac polypeptides completely inhibited endothelial migration to repair the wounds; whereas, transduction with control β-galactosidase particles had no effect (Fig. 2e & 2f).
Two alternative approaches were employed to confirm the capability of the S1P1/Akt/Rac pathway in regulating cell motility to repair the endothelial injury. As shown in Fig. 3a, S1P significantly enhanced endothelial migration in the transwell migration assay, and S1P-induced chemotaxis was markedly reduced in S1P1 knockdown endothelial cells. Moreover, transduction with dominant-negative Akt and Rac completely abrogated S1P-induced chemotactic response (Fig. 3b). In a control, transduction with β-galactosidase had no effect on S1P-enhanced endothelial chemotaxis. In addition, a small scrape wound was made across a confluent monolayer of HUVEC and cell migration into the denuded area was measured (Fig. 3c). S1P treatment significantly accelerated cell migration into the wounded area; whereas, wound closure in the absence of S1P was retarded (Fig. 3c, upper panels). Furthermore, the denuded area remained unpopulated in dominant-negative Akt and Rac transduced cells after S1P stimulation (Fig. 3c, lower panels). In a control, the denuded area was repopulated by β-galactosidase transduced endothelial cells in the presence of S1P treatment (Fig. 3c, middle panel). These results together suggest that the S1P-activated S1P1/Akt/Rac pathway is capable of enhancing cell motility to repair the injured endothelial monolayer.

We next utilized the ECIS assay to examine the effects of S1P on cell-cell interaction in confluent endothelial monolayers. As shown in Fig. 4a, a robust increase of TEER was observed immediately after S1P treatment, indicating that S1P was able to enhance endothelial cell-cell interaction. The S1P-induced cell-cell interaction was sustained for more than 6-10 hours after S1P treatment and thus was not a transient event. S1P-enhanced TEER was markedly abrogated in S1P1 knockdown endothelial cells (si-S1P1), and not in control cells stably expressing si-RNA for luciferase (si-Luc) (Fig. 4b). This indicates that S1P-enhanced TEER is primarily controlled by the endothelial S1P1 receptor. This conclusion was supported by the observation that S1P-enhanced endothelial barrier integrity was abrogated in the presence of pertussis toxin (PTx, Fig. 4c), as S1P1 signaling is dependent on the G α heterotrimeric G protein. Previously it was shown that S1P1 signaling activates the PI-3 kinase (PI3K)/Akt/ Rac pathway to regulate endothelial chemotaxis and morphogenesis (18). Therefore, we examined whether PI3K/Akt/Rac signaling controls S1P-mediated TEER. As shown in Fig. 4d, treatment with Ly294002, an inhibitor of PI3K, or adenoviral particles carrying dominant-negative Akt or Rac constructs, significantly inhibited the S1P-enhanced TEER. Together, these results indicate that S1P-enhanced intercellular interaction is controlled by the S1P1-mediated G i/ PI3K/ Akt/ Rac signaling pathway.

S1P-induced increase in TEER is a consequence of enhanced barrier integrity in pulmonary endothelial cells (10, 11). Because tight junctions are important in regulating barrier integrity, we examined whether S1P stimulates the formation of tight junctions in endothelial cells. Using electron microscopy analysis, we determined that S1P markedly enhanced the formation of tight junctions in neighboring endothelial cells (arrows, Fig. 4e). Tight junctions were quantitated in endothelial cells treated without or with S1P. Approximately 15 – 40 electron microscopic sections were examined in each sample. In S1P-treated endothelial cells, tight junction structures were identified in 72.06 ± 11% of electron microscopic sections; whereas, tight junctions were identified in 50.51 ± 4.5% of the examined sections in control endothelial cells (lower panel, Fig. 4e). This result indicates that the observed S1P-dependent increase in monolayer TEER is due at least in part to a stimulation of tight junction formation.
To investigate the molecular details of S1P-regulated integrity function and migratory response, endothelial cells treated without or with S1P were immunostained with antibodies for proteins functionally involved in cell-cell interaction and chemotaxis. These included Zonula Occludens-1 (ZO-1), claudin-5, Junctional adhesion molecule (JAM), platelet/endothelial cell adhesion molecule-1 (PECAM), Vascular/endothelial-cadherin (VE-cad), and cortactin. As shown in Fig. 5, PECAM, JAM, and VE-cad were significantly and exclusively re-distributed to cell-cell junction areas after S1P stimulation (arrows, Fig. 5), suggesting that these molecules may be involved in regulation of endothelial cell-cell interaction, but not functions at other sites, and thus contribute to S1P-enhanced endothelial integrity function. In contrast, ZO-1 and claudin-5 were translocated to both cell-cell contact areas and migratory fronts in S1P-treated ECs (Fig. 5 & 6a). Cortactin polypeptide was re-distributed to lamellipodia after S1P stimulation (Fig. 6b). This observation implies that ZO-1 and claudin-5 may be functionally important in regulating both endothelial barrier integrity and migratory response.

Interestingly, ZO-1 polypeptides were shown to be colocalized with cortical actin in the leading edges of migrating cells after S1P stimulation (arrowheads, Fig. 6a). It should be noted that the colocalization of ZO-1 and cortical actin was only observed at the cell migratory fronts and not in the cell junctional regions (arrows, lower panels, Fig. 6a). Because cortactin is an essential component in the formation of cellular cortical actin (20, 21) and also because ZO-1 was shown to interact with cortactin (22), we examined whether S1P-mediated ZO-1 translocation to the lamellipodia is dependent on cortactin polypeptide. This notion was supported by the observation that cortactin and ZO-1 polypeptides were co-localized in the lamellipodia (arrows, Fig. 6b), but not in the cellular junctions (arrowheads, Fig. 6b), in S1P-treated endothelial cells.

Previously, it was shown that the discs large (dlg)-like domain in the N-terminal half of ZO-1 polypeptide interacts with α-catenin (23). Therefore, we determined whether the S1P-mediated ZO-1 translocation to endothelial cell-cell contact regions is mediated by α-catenin association. As shown in Fig. 6c, S1P strongly stimulated the formation of zigzag-like junctional structures in endothelial cells. ZO-1 and α-catenin polypeptides were exactly colocalized in the newly formed junctional structures (arrows in Fig. 6c). This result suggests that the S1P-induced ZO-1 redistribution to endothelial junctional regions might be mediated at least in part by a α-catenin-dependent mechanism.

Next, we investigated the mechanisms of S1P-mediated lamellipodia localization of ZO-1 polypeptides. It has been demonstrated that ZO-1 interacts with the SH3 domain of cortactin via its C-terminal proline-rich region (22). Therefore, we predicted that ZO-1 lacking this region should be deficient in redistribution to lamellipodia. We utilized CHO cells as a working model, because HUVECs are very difficult to transfect with plasmid DNA. As shown in Fig. 7a, the myc-tagged full length ZO-1 polypeptides, when expressed in CHO cells stably expressing the S1P1 receptor (18), were translocated to the lamellipodia following S1P stimulation (arrows in top panels). Similarly, the S1P stimulated myc-tagged C-terminal half of ZO-1 (ZO-1(CH)) redistributed to the leading edges of the migrating cells (arrows in middle panels, Fig. 7a). In contrast, the N-terminal half of the ZO-1 was unable to relocate to cell cortical actin areas in S1P-treated CHO/S1P1 cells (lower panels, Fig. 7a), despite the fact that lamellipodia were clearly formed (arrows in lower right panel, Fig. 7a). In addition, we knocked-down endothelial cortactin with cortactin antisense phosphothioate oligonucleotide (PTO), and examined the
spatial behavior of ZO-1 in cortactin knockdown endothelial cells. As shown in Fig. 7b, transfection of cortactin antisense PTO markedly diminished endogenous cortactin (41 ± 16% reduction, n=2) in human brain microvascular endothelial cells (HBMEC). Immunofluorescent analysis showed that S1P was unable to induce lamellipodia formation and cortactin translocation in antisense PTO transfected cells (arrowhead in lower left panel, Fig. 7c). Furthermore, ZO-1 polypeptides were undetected in the migratory fronts (arrowhead, lower right panel) but were able to relocate to cell-cell junctional regions (black arrow, lower right panel) in antisense PTO transfected cells after S1P stimulation. Together, it suggests that ZO-1, a polypeptide well documented in regulating cell-cell junctions, may also play a critical role in controlling endothelial migration via a cortactin-dependent mechanism.

We next examined whether S1P-induced ZO-1 redistribution was governed by the S1P1/ Gi/ Akt/ Rac signaling pathway, which was demonstrated to be crucial in regulating S1P-mediated endothelial migratory response and barrier integrity (Fig. 2-4). The S1P-mediated redistribution of ZO-1 to the junctional regions and lamellipodia was markedly reduced by pertussis toxin treatment (PTx) and dominant-negative Akt expression in HUVECs (Fig. 8). Akt-mediated phosphorylation at Thr-236 residue of S1P1 receptor is indispensable for S1P-induced Rac activation, endothelial migration, and morphogenesis (18). Here, we observed that Akt-mediated S1P1 receptor phosphorylation was a prerequisite for S1P-induced ZO-1 translocation, because the expression of the Akt phosphorylation-deficient S1P1 mutant (S1P1T236A) (18) inhibited ZO-1 redistribution to endothelial junctions and lamellipodia. Furthermore, the redistribution of ZO-1 was significantly abrogated by the expression of dominant negative -Cdc42 and -Rac GTPases. Therefore, the translocation of ZO-1 to both the endothelial junctional regions and migratory edges in S1P-stimulated endothelial cells is mediated by the Gi/ Akt/ S1P1 phosphorylation/ Cdc42 and Rac signaling cascade.

To further confirm the role of ZO-1 polypeptides in endothelial barrier integrity and chemotactic response, ZO-1 stably knocked-down endothelial cells were established by the Lentiviral-mediated si-RNA oligonucleotides technique. As shown in Fig. 9a, ZO-1 expression was significantly diminished in endothelial cells stably expressing si-ZO-1 constructs #1 and #2; whereas, si-Luciferase (si-Luc) had no effect. ZO-1 knockdown appears to be specific as the cortactin protein remained intact in si-ZO-1 cells. S1P-mediated endothelial chemotaxis was significantly abrogated in ZO-1 knockdown cells (Fig. 9b). Also, both S1P-mediated endothelial barrier integrity and migratory response in ECIS assays were markedly diminished in ZO-1 knockdown endothelial cells, compared to the control si-Luc cells (Fig 9c & 9d). Together, these results strongly suggest that ZO-1 polypeptide plays a critical role in controlling endothelial barrier integrity and chemotactic responses.

Discussion

By utilizing ECIS analysis on electrically injured or confluent endothelial cultures, S1P treatment was shown to significantly enhance the endothelial migration and barrier integrity (Fig. 2 & 4). These two S1P-mediated activities are regulated by the S1P1/ Akt/ Rac small GTPase pathway, which was previously demonstrated to play a critical role in regulating S1P-induced cytoskeletal remodeling and morphogenesis (14, 18). It suggests that the regulatory molecule(s) down-stream of S1P/ Akt/ Rac signaling may be present to control these two endothelial activities. This
regulatory molecule should be activated at both cell-cell junctions and migratory fronts in S1P-stimulated endothelial cells. To identify this molecule, the cellular localizations of several candidate molecules were examined in endothelial cells with or without S1P treatment. Among them, VE-cadherin, PECAM and JAM were observed only in endothelial junctional areas (Fig. 5), suggesting they may be only functionally involved in S1P-induced cell-cell interactions and barrier integrity. In contrast, ZO-1 and Claudin-5, well characterized molecules in the formation of tight junctions, were redistributed to both endothelial junctional regions and the lamellipodia. Furthermore, the S1P-stimulated ZO-1 redistribution to both endothelial junctions and lamellipodia was mediated by the S1P1 / Gi/ Akt/ Rac pathway (Fig. 8). Together, these results suggest tight junction associated protein ZO-1 is one of the missing links in S1P-regulated endothelial chemotaxis and barrier integrity. This notion was supported by the fact that S1P-mediated endothelial migration and barrier integrity were markedly inhibited in ZO-1 knockdown endothelial cells (Fig. 9).

ZO-1 was first described as a component of epithelial tight junctions (24, 25); it was also identified in nonepithelial cells, where it interacts with adherens junctions (23, 26). Moreover, ZO-1 was shown to associate with connexin43 to regulate the plaque size and organization of gap junctions (27-29). Gap junctions are abundant in endothelial cells. However, we did not observe any detectable redistribution of connexin32 and connexin43 in endothelial cells after S1P stimulation (data not shown), suggesting that gap junctions may not contribute to S1P-induced TEER. Furthermore, we demonstrate that in addition to ZO-1, S1P also induced redistribution of Claudin-5, a well-characterized tight junctional polypeptide, to endothelial junctional areas (Fig. 5). Moreover, examinations with electron microscope directly demonstrated that S1P treatment stimulated tight junction formation (Fig. 4e). Because barrier integrity function is primarily mediated by intercellular tight junctions; the result of this study demonstrates for the first time that S1P/ S1P1 signaling is capable of regulating endothelial barrier integrity by stimulating the formation of tight junctions in endothelial cells.

It should be noted that the baseline transendothelial electrical resistances (TEERs) in si-S1P1 and si-ZO-1 were significantly less than those in si-Luc and control endothelial cells (Fig. 2c, 4b, 9c, & 9d). The baseline TEERs in si-S1P1 (#23), si-S1P1 (#24), si-ZO-1 (#1), and si-ZO-1 (#2) are 69.3 ± 1.5%, 72.6 ± 0.8%, 76.6 ± 1.2%, and 81.8 ± 0.7%, respectively, of those in parental or si-Luc endothelial cells (Mean ± S.E. of TEERs in 24 hrs period prior to S1P addition, n=4). It suggests that S1P1 and ZO-1 are important in maintaining endothelial barrier integrity. In addition, the delta-TEERs (difference between S1P-treated and –untreated) in barrier integrity (Fig. 4b & 9c) in si-S1P1 (#23), si-S1P1 (#24), si-ZO-1 (#1), and si-ZO-1 (#2) are 41.2 ± 16.6%, 56.8 ± 12.3%, 52.7 ± 6.7%, and 56.6 ± 7.5%, respectively, of those in parental or si-Luc endothelial cells (Mean ± S.E. of delta-TEERs in 4 hrs period after S1P addition, n=4). Moreover, the delta-TEERs in migratory response (Fig. 2c & 9d) in si-S1P1 (#23), si-S1P1 (#24), si-ZO-1 (#1), and si-ZO-1 (#2) are 40.6 ± 12%, 46.2 ± 8%, 36 ± 17%, and 58 ± 11%, respectively, of those in control endothelial cells (Mean ±S.E. of delta-TEERs in 4 hrs period after S1P stimulation, n=4). Therefore, these results not only indicate that the diminished S1P-enhanced barrier integrity and migratory response in si-S1P1 and si-ZO-1 endothelial cells is not due to the reduced baseline TEER, but also suggest the crucial roles of S1P1 and ZO-1 in controlling endothelial barrier integrity and migratory response.
Cortactin, an actin-binding polypeptide, redistributes to the leading edges of migratory endothelial cells and plays a critical role in S1P-mediated chemotaxis (11, 30, 31). Evidence presented in this study show that S1P induces ZO-1 and cortactin colocalization at endothelial lamellipodia (Fig. 6b), lamellipodia translocation of ZO-1 requires C-terminal half of ZO-1 which contains the cortactin-interacting domain (Fig. 7a), and cortactin knockdown inhibits S1P-induced lamellipodia relocation of ZO-1 (Fig. 7c). Furthermore, ZO-1 knockdown significantly abrogates S1P-stimulated endothelial chemotactic response (Fig. 9b & 9d). Collectively, this data suggests that the lamellipodia localization of ZO-1 may be mediated by a cortactin-dependent mechanism and play a critical role in S1P-induced endothelial migratory response. However, the molecular mechanism underlying ZO-1 lamellipodia localization in regulating endothelial migration is not understood. Previously, it was shown that cortactin and Arp 2/3 complexes translocate to endothelial migratory fronts after S1P stimulation (31). Therefore, the lamellipodia localization of ZO-1 may modulate cortactin interacting partners and thus regulate the cytoskeletal architecture at the leading edges of migratory endothelial cells.

The cortactin polypeptide was not colocalized with ZO-1 in endothelial junctional regions (Fig. 6b), suggesting that S1P-mediated ZO-1 redistribution to endothelial cell-cell contact areas and the formation of endothelial junctions are mediated by a cortactin-independent mechanism. The molecular details of S1P-induced ZO-1 localization in the cellular junctions are currently unknown. ZO-1 and α-catenin interact directly (23, 32, 33) and the discs large (dlg)-like domain in the N-terminal half of the ZO-1 polypeptide is required for α-catenin association (23). In this study, we observed that S1P treatment resulted in the ZO-1 and α-catenin polypeptides being markedly redistributed to endothelial cell-cell junctional areas, where these two polypeptides were found to be colocalized (Fig. 6c). Therefore, this suggests that the redistribution of ZO-1 to endothelial junctional regions may be, at least in part, mediated by a mechanism involving α-catenin, and is independent of cortactin.

ZO-1 and α-catenin are two important components in the formation of tight junctions and adherens junctions, respectively (23, 32, 33). Previously, it was shown that the S1P-activated Rho family small GTPases enhanced VE-cadherin based adherens junction formation in endothelial cells (14). In this study, we demonstrated that S1P stimulation markedly enhanced the formation of endothelial tight junctions (Fig. 4e). The observed ZO-1 and α-catenin colocalization in junctional regions implies that tight junctions and adherens junctions may be cross-regulated and may concertedly mediate S1P functions in vasculature. Furthermore, PECAM and JAM polypeptides were significantly enriched in cell-cell contact areas in S1P-treated endothelial cells (Fig. 5). Thus, S1P-regulated endothelial barrier integrity function may be a biological manifestation of concertedly regulated events by various cell-cell interaction machineries, e.g. tight junctions, adherens junctions, PECAM- and JAM-mediated intercellular interaction.

References
1. Fenstermacher, J. D., Nagaraja, T., and Davies, K. R. (2000) Blood brain barrier: drug delivery and brain pathology. New York: Kluwer Academic–Plenum Publishers.
2. Gumbleton, M., and Audus, K. L. (2001) J. Pharm. Sci. 90, 1681–1698
3. Plumb, J., McQuaid, S., Mirakhur, M., and Kirk, J. (2002) *Brain Pathol.* **12**, 154–169
4. Rosenberg, G. A. (2002) *Neuroscientist* **8**, 586–595
5. Kalaria, R. N. (2002) *Cerebrovasc. Dis.* **13**, 48–52
6. Miyakawa, T. (2002) *Ann. NY Acad. Sci.* **977**, 303–305
7. Andersson, L. M., Hagberg, L., Fuchs, D., Svennerholm, B., and Gisslen, M. (2001) *J. Neurovirol.* **7**, 542–547
8. Bussolino, F., Mitola, S., Serini, G., Barillari, G., and Ensoli, B. (2001) *Int. J. Biochem. Cell Biol.* **33**, 371–390
9. Yatomi, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1995). *Blood.* **86**, 193-202
10. Garcia, J. G. N., Liu, F., Verin, A. D., Birukova, A., Dechert, M. A., Gerthoffer, W. T., Bamburg, J. R., and English, D. (2001) *J. Clin. Invest.* **108**, 689–701
11. Dudek, S. M., Jacobson, J. R., Chiang, E. T., Birukov, K. G., Wang, P., Zhan, X., and Garcia, J. G. (2004) *J. Biol. Chem.* **279**, 24692-24700
12. Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (1998) *J. Biol. Chem.* **273**, 29745–29753
13. Huber, J. D., Egleton, R. D., and Davis, T. P. (2001) *Trends Neurosci.* **24**, 719–725
14. Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M. Sha’afi, R. I., and Hla, T. (1999). *Cell* **99**, 301-312
15. Lee, M. J., VanBrocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzelev, R., Spiegel, S. and Hla, T. (1998). *Science.* **279**, 1552-1555
16. Krump-Konvalinkova, V., Yasuda, S., Rubic T., Makarova N., Mages, J., Erl, W., Vosseler, C., Kirkpatrick, J., Tigygi, G., and Siess W. (2005) *Arterioscler. Thromb. Vasc. Biol.* **25**, 1-7
17. Keese, C. R., Wegener, J., Walker, S. R., and Giaever, I. (2004) *Proc. Natl. Acad. Sci. USA.* **101**, 1554-1559
18. Lee, M. J., Thangada, S., Paik, J. H., Gopal, S. P., Ancellin, N., Chan, S. S., Wu, M., Morales-Ruiz, M, Sessa, W. C., Alessi, D. and Hla, T. (2001) *Mol Cell.* **8**, 693-704
19. Kalman, D., Gomperts, S. N., Hardy, S., Kitamura, M., and Bishop, J. M. (1999) *Mol Biol Cell.* **10**, 1665-1683
20. Weed, S. A., Karginov, A. V., Schafer, D. A, Weaver, A. M., Kinley, A. W., Cooper, J. A., Parsons, J. T. (2000) *J. Cell Biol.* **151**, 29-40
21. Urano, T., Liu, J., Zhang, P., Fan, Y. X., Egile, C., Li, R., Mueller, S. C., Zhan, X. (2001) *Nat. Cell Biol.* **3**, 259-266.
22. Katsube, T., Takahisa, M., Ueda, R., Hashimoto, N., Kobayashi, M., and Togashi, S. (1998) *J Biol. Chem.* **273**, 29672-29677
23. Itoh, M., Nagafuchi, A., Moroi, S., and Tsukita, S. (1997) *J Cell Biol.* **138**, 181-192
24. Stevenson, B. R., Siliciano, J. D., Mooseker, M. S., Goodenough, D. A. (1986) *J. Cell Biol.* **103**, 755-766
25. Anderson, J. M., Stevenson, B. R., Jesaitism, L. A., Goodenough, D. A., Mooseker, M. S. (1998) *J. Cell Biol.* **106**, 1141-1149
26. Yokoyama, S., Tachibana, K., Nakanishi, H., Yamamoto, Y., Irie, K., Mandai, K., Nagafuchi, A., Morden, M., Takai, Y. (2001) *Mol. Biol. Cell* **12**, 1595-1609
27. Giepmans, B. N., Moolenaar, W. H. (1998) *Curr. Biol.* **8**, 931-934
28. Hunter, A. W., Barker, R. J., Zhu, C., Gourdie, R. G. (2005) *Mol. Biol. Cell* **16**, 5686-98
29. Zhu, C., Barker, R. J., Hunter, A. W., Zhang, Y., Jourdan, J., Gourdie, R. G. (2005) *Microsc. Microanal.* **11**, 244-248
30. Li, Y., Uruno, T., Haudenschild, C., Dudek, S. M., Garcia, J. G., and Zhan, X. (2004) *Exp. Cell Res.* **298**, 107-121
31. Lee, J. F., Ozaki, H., Zhan, X., Wang, E., Hla, T., Lee, M. J. (2006) *Histochem. Cell Biol.* **14**, 1-8
32. Muller, S. L., Portwich, M., Schmidt, A., Utepbergenov, D. I., Huber, O., Blasig, I. E., and Krause, G. (2005) *J. Biol. Chem.* **280**, 3747-3756
33. Smalley, K. S., Brafford, P., Haass, N. K., Brandner, J. M., Brown, E., and Herlyn, M. (2005) *Am. J. Pathol.* **166**, 1541-1554

**Figure Legend**

*Fig. 1. Real-time measurement of endothelial migration with ECIS assay.* (a) HUVECs were plated into each well of 8W1E arrays and incubated at 37°C to allow the TEER to reach equilibrium. Twenty-two hours later, the cells attached to the center microelectrodes were killed by applying 5 volts electrical current for 30 second (arrowhead), which resulted in an abrupt impedance drop. Subsequently, endothelial migratory responses were determined in real-time by measuring the recovery of TEER, an indicator of endothelial migration into the wounded area. (b) Micrographs were taken from ECIS arrays before (panel I), immediately after (panel II), 2 hrs after (panel III), and 4 hrs after (panel IV) wounding (arrows in *Fig. 1a* show the corresponding time points). Note that the high electrical current completely killed endothelial cells attached to the microelectrode (panel II), and the rise of TEER was a result of the surrounding viable cells migrating into the wounded electrodes (panels III, IV). The micrographs are representative of 4 ECIS wells at each time point. Scale bar, 125 µM.

*Fig. 2. S1P accelerates endothelial migration in ECIS wounding assay.* (a) Endothelial cells on the microelectrodes of the ECIS wells were killed with a high electrical voltage as described. Subsequently, the migration of viable cells into the wounded microelectrodes was measured in real-time by electrical impedance in the presence or absence of S1P (500 nM). (b) Micrographs of ECIS arrays, taken at 3 hrs after electrical injury. –S1P and +S1P, without or with S1P addition, respectively. Scale bar, 125 µM. (c) Cell migration in ECIS wounding assays was performed in endothelial cells stably transduced with si-RNA for S1P1 (si-S1P1) or Luciferase (si-Luc) with or without S1P stimulation. (d) Endothelial cells were transduced with adenoviral particles carrying β-galactosidase (β-gal), dominant negative -Akt (dnAkt), and -Rac (dnRac) cDNAs as described (200 m.o.i. each) (18). After killing the cells on the microelectrodes, the migratory responses were measured with or without S1P treatment. (e) 50 µg of extracts from endothelial cells transduced with si-Luc and two different si-S1P constructs (#23 and #24) were directly immunoblotted with anti-S1P1 (upper panel). Lower panel, 500 µg of extracts were immunoprecipitated with anti-S1P1 followed by immunoblotting with anti-S1P1. Densitometric Quantitation showed that ~80% and 60% of S1P1 were knocked-down in si-S1P1 #23 and #24 transduced endothelial cells. Middle panel, Western-blot with anti-actin showed the protein equal loading and the absence of off-target effect by si-S1P1 silencing. (f) In a parallel experiment, endothelial cells were transduced with 200 m.o.i of adenoviral particles as described in panel d. Five hundred micrograms of cell extracts were immunoprecipitated with anti-S1P1, followed by immunoblotting with anti-S1P1 (top panel). Alternatively, 50 µg of extracts were immunoblotted with anti-Akt or anti-myc to show the expression of transduced polypeptides (middle and lower panels) (18, 19). Left lane, without endothelial extracts. The arrows in panels a, c, and d are when cells on the microelectrodes were killed by elevating the voltage, followed...
by stimulation with or without S1P. Panels a, c, and d are Mean ± S.E. of two determinants from a representative experiment, which has been repeated at least three times with identical results.

**Fig. 3.** S1P mediated signaling regulates endothelial migration. (a) Chemotactic responses were measured by transwell migration assay as described in Experimental procedures. Note that S1P-induced chemotactic responses were significantly inhibited in both construct #23 and #24 si-S1P1 endothelial cells. (b) Transwell migration assays were performed with ECs transduced with Adenoviral particles carrying β-galactosidase, dominant-negative -Akt and -Rac vectors in the presence or absence of S1P stimulation. The data in a and b are the Mean ± S.E. of three determinants from a representative experiment, which was repeated two times with similar results. (c) Phase-contrast micrographs show the S1P-enhanced repair of endothelial injuries. Wounds were introduced in confluent monolayers of wild-type (upper panels), β-galactosidase (middle panels), dominant-negative -Akt or –Rac (lower panels) transduced cells with sterile microtips. After stimulation without or with S1P for 16 hrs, cells were fixed. The distances (numbers in upper right corners, µm) between two edges of wounds were quantitated with a Zeiss microscope equipped with Axiovert image software (Carl Zeiss). The data is the Mean ± S.E. of 6 microscopic fields from two determinants, which were repeated three times with similar results.

**Fig. 4.** S1P signaling enhances transendothelial electrical resistance and induces tight junction formation. (a) The TEER, an indicator of endothelial integrity function (10, 17), was measured in real-time in confluent endothelial cultures treated without or with S1P (500 nM). (b) The TEER was determined in si-S1P1 and si-Luc cells in the presence or absence of S1P. (c) HUVEC cells were pretreated with or without pertussis toxin (PTx, 100 ng/ ml, 1 hr). After stimulation with S1P, TEER was measured in real-time. (d) Cells were pretreated with Ly294002 (10 µM, 30 min), β-gal, dnAkt, and dnRac adenoviral particles (200 m.o.i. each). After stimulation without or with S1P, the TEER was measured. Panels a, b, c, and d are Mean ± S.E. of two determinants from a representative experiment, which has been repeated at least three times with identical results. (e) Electron microscopic analysis shows S1P induced tight junction formation in HUVECs (arrows). Scale bar, 0.226 µm. Lower panel, random sections of control or S1P-treated samples were quantitated for tight junctions under electron microscope. Data represents the Mean ± S.E. from two samples of control or S1P-treated HUVECs, and ~ 15 – 40 sections were examined in each sample. **: p < 0.001 (t-test).

**Fig. 5.** S1P induces translocation of junctional or adhesion molecules. HUVEC cells were treated without or with S1P (500 nM, 30 min) and the cellular localization of the junctional or adhesion molecules were visualized by immunostaining. Note that platelet/endothelial cell adhesion molecule-1 (PECAM), junctional adhesion molecule (JAM), and vascular/endothelial-cadherin (VE-cad) were markedly and exclusively relocated to cell-cell junctional areas after S1P treatment. In contrast, claudin-5, a tight junction molecule, was observed in both the lamellipodia and junctional regions. Arrows, cell-cell contacts; arrowheads, migratory fronts. Scale bar = 18 µm.

**Fig. 6.** S1P induces colocalization of ZO-1/ cortactin in the lamellipodia and ZO-1/ α-catenin in junctional areas. (a) After stimulation without or with S1P (500 nM, 30 min), HUVECs were doubly stained with anti-ZO-1 (Zymed) and Phalloidin. Note that S1P significantly induced the
formation of cellular junctions (arrows), and markedly relocated ZO-1 to cortical (arrowheads) and junctional (arrows) regions. (b) HUVEC cells were doubly stained with rabbit anti-ZO-1 and mouse anti-cortactin (Transduction Laboratory) in the absence or presence of S1P stimulation. Note that cortactin and ZO-1 polypeptides were colocalized in the S1P-induced lamellipodia, whereas cortactin was not observed in S1P-induced junctional regions. (c) Endothelial cells were stimulated without or with S1P, the subcellular localization of ZO-1 and α-catenin was detected by immunostaining with rabbit anti-ZO-1 (Zymed) and goat anti-α-catenin (Santa Cruz). Note that ZO-1 and α-catenin were completely colocalized in S1P-induced endothelial junctional regions (arrows). Scale bars in a, b, and c are 26, 32, and 36 µm, respectively.

Fig. 7. S1P induces ZO-1 relocation to the lamellipodia by a cortactin-dependent mechanism. (a) CHO cells stably expressing the S1P1 receptor were transfected with the myc-tagged full-length (FL), C-terminal half containing cortactin interacting domain (CH), or N-terminal half without cortactin interacting domain (NH) of ZO-1 cDNAs (23). After S1P stimulation, cells were immunostained with anti-myc (left panels) and phalloidin (right panels) to show the ectopically expressed ZO-1 polypeptides and actin structures, respectively. Note that the myc-tagged full-length and C-terminal half of ZO-1 polypeptides were redistributed to the lamellipodia (arrows in upper and middle panels), whereas the N-terminal half of ZO-1 was unable to relocate to the lamellipodia. Scale bar = 21 µm. (b) HBMECs were co-transfected with sense (S-Cort.) or anti-sense (αS-Cort.) phosphothioate oligonucleotide (PTO, 100 nM), along with GFP cDNA by lipofectAime reagent. Western-blotting analysis shows that anti-sense PTO treatment results in a 41 ± 16% reduction of cortactin (Mean ± S.E. of two determinants); whereas it does not have non-specific effect on ZO-1 polypeptides. (c) HBMECs were cotransfected with cortactin antisense PTO and GFP cDNA vector. After S1P stimulation, cells were immuno-stained with cortactin or ZO-1 antibody (lower panels). Upper panels, the transfected cells were identified by the expression of GFP polypeptides. Note that S1P treatment resulted in lamellipodia formation in the untransfected cells (white arrows). Also note that cortactin and ZO-1 were relocated to lamellipodia in the untransfected cells (white arrows). In contrast, neither lamellipodia formation nor translocation of cortactin and ZO-1 to lamellipodia was observed in antisense PTO transfected cell (arrowheads, lower left and right panels). However, S1P was able to redistribute ZO-1 to zigzag-like junctional structures in antisense PTO transfected cells (black arrow, lower right panel). Scale bar = 18 µm.

Fig. 8. S1P-induced ZO-1 redistribution to both the lamellipodia and cell-cell contact regions is mediated by the G/ Akt/ S1P1 phosphorylation/ Cdc42-Rac pathway. HUVEC cells were pretreated with PTx (100 ng/ ml, 1 hr), adenoviral particles carrying dominant negative –Akt (dnAkt), -Cdc42 (dnCdc42), -Rac (dnRac), or Akt phosphorylation-deficient S1P1 mutant (S1P1T236A) (200 m.o.i. each), as described (18). After S1P stimulation, the cellular localization of the ZO-1 polypeptides was visualized by immunostaining with ZO-1 antibody. Note that S1P-induced ZO-1 redistribution to both cell-cell contacts (arrows) and the lamellipodia (arrowheads) was markedly abrogated by the treatments. Scale bar = 26 µm.

Fig. 9. Diminished integrity function and chemotactic responses in ZO-1 knockdown endothelial cells. (a) HUVECs were stably transduced with Lentiviral particles carrying si-RNA oligonucleotides for Luciferase or two different oligonucleotides for ZO-1 (construct #1 and #2).
Cell extracts were Western-blotted with anti-ZO-1 (upper panel) and anti-cortactin (lower panel). Note that the expression of ZO-1 polypeptides in HUVECs stably transduced with si-ZO-1 constructs was significantly inhibited, whereas the expression of cortactin was not affected. Data are Mean ± S.E. of four determinants. (b) Chemotactic responses were performed with transwell migration assay as described in Experimental procedures. Note that the S1P-induced chemotactic responses were significantly inhibited in both construct #1 and #2 si-ZO-1 endothelial cells. The figure is the Mean ± S.E. of three determinants from a representative experiment, which was repeated two times with similar results. S1P-induced endothelial integrity (c) and chemotactic response (d), measured by ECIS assays, were markedly reduced in si-ZO-1 knockdown endothelial cells. The arrow in panel c indicates the addition of S1P (500 nM), and in panel d indicates when cells were killed by elevating the voltage and treated with S1P. Panels c and d are Mean ± S.E. of two determinants from a representative experiment, which has been repeated at least three times with identical results.

Acknowledgement: We thank Dr. Binks Wattenberg for the critical comments, Dr. Shoichiro Tsukita (Kyoto University) for myc-tagged ZO-1 cDNAs, Dr. Kwang Sik Kim (Johns Hopkins University) for HBMECs, and the Electron Microscope Facility of University of Connecticut Health Center for the EM analysis. This work is supported by NIH grants R01HL071071 (M.L), R01HL067330 (T.H), and DoD grants 3R37AG007444 (E.W). M.L. is a recipient of the New Investigator Award of Gheens Foundation, Louisville, KY, USA.

Abbreviation: S1P, sphingosine-1-phosphate; S1P1 (old nomenclature EDG-1, endothelial differentiating gene-1), the high affinity GPCR for S1P; ZO-1, Zonula Occludens-1; ECIS, electrical cell-substrata impedance sensing technique; GPCR, G-protein coupled receptor; si-S1P1, silencing RNA for S1P1 receptor; si-ZO-1, silencing RNA for ZO-1 polypeptide.
Lee et. al., Fig. 1
Lee et. al., Fig. 2
Lee et. al., Fig. 3
Lee et. al., Fig. 4
Lee et. al., Fig. 5
Lee et. al., Fig. 6
Lee et. al., Fig. 7
Lee et. al., Fig. 8
Lee et. al., Fig. 9