The E3 ubiquitin ligase MARCH3 controls the endothelial barrier

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Cell–cell contacts coordinate the endothelial barrier function in response to external cues. To identify new mediators involved in cytokine-promoted endothelial permeability, we screened a siRNA library targeting E3 ubiquitin ligases. Here, we report that silencing of the late endosome/lysosomal membrane-associated RING-CH-3 (MARCH3) enzyme protects the endothelial barrier. Furthermore, transcriptome analysis unmasked the upregulation of the tight junction-encoding gene occludin (OCLN) in MARCH3-depleted cells. Indeed, MARCH3 silencing results in the strengthening of cell-cell contacts, as evidenced by the accumulation of junctional proteins. From a molecular standpoint, the FoxO1 forkhead transcription repressor was inactivated in the absence of MARCH3. This provides a possible molecular link between MARCH3 and the signaling pathway involved in regulating the expression of junctional proteins and barrier integrity.

Keywords: claudin-5; FoxO; occludin; permeability; tight junction; ubiquitin

The vascular endothelium forms a semipermeable selective barrier that allows the controlled passage of fluids, molecules and cells to and from the bloodstream. This function relies on the organization of cell–cell junctions, orchestrated notably through VE-cadherin-based adherens junctions (AJs) [1,2]. Angiogenic and inflammatory factors elevate endothelial permeability and operate through various signaling pathways; this can involve both transcriptional control of VE-cadherin and post-translational modifications of AJ-enriched proteins, such as phosphorylation of VE-cadherin and its partners, VE-cadherin internalization, and cytoskeleton contractility [1,3–8]. Of note, VE-cadherin expression and trafficking control AJ organization, as well as composition of tight junctions (TJs) and vice versa [9,10]. Indeed, claudin-5-dependent TJs are not functional in VE-cadherin (CDH5)-null endothelial cells [9,11]. From a molecular standpoint, VE-cadherin sustains the activation of the PI-3 kinase (PI3K)/AKT pathway, and the subsequent phosphorylation of the forkhead repressor transcription factor FoxO1, leading to its inhibition [9,11]. Conversely, in cells lacking VE-cadherin, nonphosphorylated active FoxO1 accumulated within the nucleus, where CLDN5 gene expression is constitutively silenced. Consequently, this effect was lost upon rescue of VE-cadherin expression. Modulation of the FoxO1 pathway might, thus, coordinate the composition of AJs and TJs in endothelial cells, while involved in vascular homeostasis and endothelial proliferation [9,11–13].

Abbreviations
AJs, adherens junctions; hCMEC, human cerebral microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; MARCH3, membrane-associated RING-CH-3; PI3K, PI-3 kinase; TJs, tight junctions.
Besides phosphorylation, ubiquitylation can also take part in trafficking and degradation of adhesion molecules, as envisioned by early reports on cadherin internalization [14,15]. Proteasome-mediated degradation was indeed proposed to coordinate VE-cadherin stability and therefore AJ architecture and endothelial barrier integrity [4,15]. For instance, internalized VE-cadherin could be processed through a lysosomal/proteasome pathway [15–17]. More recently, inflammatory agents, such as bradykinin and histamine, were established to provoke VE-cadherin ubiquitylation in vitro [4]. This process was suggested to be required for VE-cadherin internalization and vascular barrier opening. Interestingly, the blood–brain barrier damage was shown to be associated with occludin ubiquitylation and degradation [18]. Furthermore, the viral transmembrane ubiquitin ligase K5 encoded by the Kaposi Sarcoma Herpes Virus, which displays some homologies with the MARCH E3 ubiquitin ligase family, was shown to down-regulate membrane stability and therefore AJ architecture and endothelial barrier permeability [18].

Although lysosomal trafficking and ubiquitylation of endothelial junctional proteins appear intertwined, the ubiquitin ligases involved in these processes are yet to be characterized. Here, we inferred that endosome/lysosome resident E3 ubiquitin ligases might contribute to endothelial barrier permeability. Therefore, we deployed a siRNA library screen that targets transmembrane E3 ubiquitin ligases and identified MARCH3 as a regulator of endothelial permeability in response to inflammatory factors.

**Materials and methods**

**Cell culture and transfections**

Human umbilical vein endothelial cells (Eahy926 HUVECs) were purchased from ATCC and maintained in DMEM plus 10% fetal bovine serum (Life Technologies, Courtaboeuf, France). Human cerebral microvascular endothelial cells (hCMEC/D3, [21]) were grown on collagen-coated plates in EBM2 complete medium (Lonza, Basel, Switzerland). Cells were transfected using Lipofectamine RNAimax (Life Technologies). hCMEC/D3 cells were seeded onto collagen-coated 3.0- and 0.4-μm pore size PTFE membrane inserts (Corning, Amsterdam, the Netherlands), respectively. The passage of FITC-labeled 40 kDa dextran (Life Technologies) was measured 30 min after IL-8 (50 ng·mL⁻¹) or histamine (100 nm) challenge using a BMG Labtech automate plate reader. Screening on IL-8 and histamine-treated cells was conducted as described previously [21]. The library consists of four nonsilencing sequences (sic) and two sequences per target [22]. Nontreated cells were included for the four sic-transfected conditions only, to serve as internal controls for histamine and IL-8 action.

**Permeability assays**

Permeability assays were performed as described previously in [21,23]. Briefly, 10⁵ transfected HUVECs and 5 × 10⁵ hCMEC/D3 cells were seeded onto collagen-coated 3.0- and 0.4-μm pore size PTFE membrane inserts (Corning, Amsterdam, the Netherlands), respectively. The passage of FITC-labeled 40 kDa dextran (Life Technologies) was measured 30 min after IL-8 (50 ng·mL⁻¹) or histamine (100 nm) challenge using a BMG Labtech automate plate reader. Screening on IL-8 and histamine-treated cells was conducted as described previously [21]. The library consists of four nonsilencing sequences (sic) and two sequences per target [22]. Nontreated cells were included for the four sic-transfected conditions only, to serve as internal controls for histamine and IL-8 action.

**Reagents and antibodies**

FoxO1 inhibitor AS1708727 was from Merck (Nottingham, UK) and used at 100 nm. Primary antibodies used were: VE-cadherin, GAPDH, and tubulin (Santa Cruz, Heidelberg, Germany), occludin and claudin-5 (Life Technologies), p-FoxO1, p-FoxO1/3, p-AKT, and AKT (Cell Signaling, Ozyme, Saint-Quentin, France). Primary antibodies were diluted at 1/1000 for western-blots. HRP-coupled antibodies were from Jackson ImmunoResearch (Suffolk, UK) (1/5000 dilution).

**Immunofluorescence**

Samples for immunofluorescence were processed as described in [24]. Briefly, samples were fixed in paraformaldehyde 4%, permeabilized in Triton 0.5% and blocked in PBS-BSA 3%. Primary antibodies were goat anti-VE-cadherin (1/200 dilution, Santa Cruz), rabbit anti-FoxO1/3 (1/100 dilution, Cell Signaling), and mouse anti-ZO1 (1/200 dilution; BD Transduction Laboratories, Le Pont-de-Claix, France). Secondary antibodies were from Life Technologies (AlexaFluor488 conjugated, 1/500 dilution). Actin cytoskeleton was stained using Alexa488-conjugated phalloidin (1/1000 dilution, BD Transduction Laboratories, Le Pont-de-Claix, France). Images were prepared with IMAGE J software and blinded postanalysis performed by two independent individuals.

**Flow cytometry**

Cells were gently scrapped and processed for flow cytometry as described in [24]. Briefly, cells were incubated at 4 °C with IgG2a PE-conjugated anti-VE-cadherin antibodies and control immunoglobulins (R&D), 10 000 events were gated on an Accuri C6 cytometer (BD Biosciences, Le Pont-de-Claix, France) and analyzed using FLOWJO.
Western-bolts and RT-PCR

Total cell lysates were prepared and processed for western blots as described in [24]. Membranes were imaged using the Fusion FX7 chemiluminescence system (Vilber, Collégien, France). RT-PCR was conducted as described in [24].

Transcriptome analysis

The expression profile of human brain endothelial cells, either control or MARCH3-depleted (2 days post-transfection) was performed with the HG-U133 Plus 2.0 Affymetrix GeneChip arrays (Genomic’ Platform; Institut Cochin, Paris, France). Supervised clustering analysis of these microarrays was then conducted using P-value > 0.05 and fold change ± 1.2. A final list of 513 genes was found down- or up-regulated (Table S2).

Results

A siRNA library screen identifies the E3 ubiquitin ligase MARCH3 in the endothelial barrier function

To identify transmembrane E3 ubiquitin ligases potentially interfering with cytokine-induced permeability, human umbilical vein endothelial cells (HUVECs) were transfected with siRNA duplexes against 46 E3 ubiquitin ligases (two sequences/target, [22]) or with non-silencing RNA (sic, four sequences) [22]. Cells were seeded on semiporous, collagen-coated membranes for permeability assays [3,25]. When knocked down, none of the tested E3 ubiquitin ligases further exacerbated IL-8- and histamine-promoted permeability (Table S1). Conversely, the silencing of nine of them (namely BFAR, MARCH2, MARCH3, MARCH6, RNF122, RNF133, RNF152, RNF175, and TRIM59) was sufficient to quell IL-8-induced permeability (Fig. 1A, left panel, green and yellow dots). Among these hits, MARCH3, RNF152, and TRIM59 were shared in the histamine-triggered permeability screen (Table S1, Fig. 1A, right panel, green and yellow dots). Based on the literature, we further focused on MARCH3 (Fig. 1A, green dots), as it has been previously linked to the endocytosis and trafficking of plasma membrane proteins [26,27]. The silencing of MARCH3 in human endothelial cells led to a significant reduction in both IL-8- and histamine-promoted permeability by around 40%, while sparing nonstimulated cells (Fig. 1A,B, Table S1). To further validate MARCH3 as a negative regulator of endothelial integrity, the permeability of human endothelial cell monolayers was next challenged using independent siRNA sequences (Fig. 1C), whose efficiency was evaluated through RT-PCR to around 60% of extinction for the most robust one (Fig. 1C). Of note, the transcript levels of MARCH2, one of the closest homologs of MARCH3, remained unaltered (Fig. 1C). In these conditions, MARCH3 silencing impaired the permeability increases in both macrovascular (HUVECs, [23]) and microvascular (hCMEC/D3, [21,28]) endothelial cells (Fig. 1D,E). Thus, our data identified MARCH3 as a lysosomal component involved in cytokine-activated endothelial permeability elevation.

MARCH3-silenced endothelial cells exhibited strengthened cell–cell junctions

Because of the prominent role of cell–cell contacts in orchestrating the endothelial barrier function [3,4,8,9], we next explored whether MARCH3 silencing impacts on endothelial junctional organization. Interestingly, neither cell viability nor cell density was affected by MARCH3 depletion (Fig. 2A,B). Furthermore, confocal analysis of 3-day old endothelial monolayers revealed that IL-8 induced an overall reorganization of phalloidin-labeled cell borders (Fig. 2C,D). However, the chemokine failed to do so in MARCH3-depleted endothelial cells (Fig. 2C,D), suggesting again that the reduction in MARCH3 expression levels opposed to IL-8, provoked junctional remodeling and subsequent increases in endothelial permeability. In keeping with this idea, immunofluorescence staining on 3-day old monolayers unveiled a discrete, but significant, increase in VE-cadherin staining intensity at the borders of adjacent cells (Fig. 2E). This was corroborated by an augmentation in surface-exposed VE-cadherin, as evaluated by flow cytometry (Fig. 2F). Thus, our data support the idea that endothelial adherens junctions are strengthened in MARCH3-silenced cells.

 Tight junctions are strengthened in MARCH3-depleted cells

To further explore the mechanisms by which MARCH3 depletion prevents permeability increases, we next conducted a gene array analysis. The transcriptome of nonsilencing control RNA-transfected human brain endothelial cells (sic) was compared to the one of MARCH3 siRNA-transfected cells. This unbiased transcriptomic analysis identified few protein encoding RNA (67% of total modified sequences) that were consistently up- (49%) or down-regulated (51%) in MARCH3-depleted cells (Table S2, Fig. 3A–C). Among the up-regulated genes was OCLN, which encodes the TJ protein occludin (Table S2, Fig. 3A–C). Indeed, RT-PCR analysis confirmed that the
siRNA-mediated down-regulation of MARCH3 was accompanied by an augmentation of OCLN transcription (Fig. 3C,D). Furthermore, CLDN5 messengers were also elevated, while CDH5 gene expression was only slightly enhanced (Fig. 3D). In agreement with this, the levels of occludin and claudin-5 TJ proteins were strongly augmented in the absence of MARCH3 (Fig. 3E). Once again, VE-cadherin expression was only mildly affected, suggesting that unlike occludin and claudin-5, the effect of MARCH3 depletion on VE-cadherin expression is likely indirect and might rather reflect a positive feedback loop, ignited through TJ stabilization (Fig. 2E,F) [9]. At the level of junctional organization, this results in reinforced TJ organization, as illustrated by augmented ZO-1 recruitment at cell borders (Fig. 3F). Thus, MARCH3 silencing, which prevents permeability increases, drives the upregulation of TJ protein expression that may ultimately culminate in the strengthening of endothelial cell–cell junctions.
MARCH3 impact on OCLN gene expression relies on the FoxO pathway

The forkhead transcription repressor factor, FoxO1, controls claudin-5 protein expression [9,13], and was more recently found involved in vascular remodeling [12]. Remarkably, MARCH3 depletion in 3-day-old starved endothelial cell monolayers was sufficient to enhance the phosphorylation of AKT and FoxO1/3, a signature for the inhibition of this transcription factor (Fig. 4A). In line with this, FoxO1 nuclear accumulation was reduced upon MARCH3 silencing, as examined by immunofluorescence-based confocal analysis (Fig. 4B). We next investigated whether the effect of MARCH3 silencing on occludin expression was mediated through FoxO1 activity. To this end, endothelial cells were challenged with the well-characterized FoxO1 inhibitor AS1708727 [29]. Interestingly, we found that FoxO1 inhibitor treatment was sufficient to elevate OCLN expression, while leaving intact MARCH3 mRNA levels (Fig. 4C). Moreover, pharmacological blockade of FoxO1 blunted histamine- and IL-8-induced permeability (Fig. 4D). As FoxO1 inhibition phenocopied MARCH3 depletion, it may act through the inhibition of the transcriptional repression activity of FoxO1, as previously demonstrated in the context of the CLDN5 promoter [9]. Collectively, our data favor a model in which MARCH3 sustains the basal activation of FoxO1 that counterpoises barrier reinforcement.

Discussion

In conclusion, our data show that the late endosome/lysosome-anchored MARCH3 E3 ligase controls the expression of the TJ proteins, occludin and claudin-5, most likely via its impact on the transcriptional repressor FoxO. Meanwhile, the overall cell–cell junctions
appeared reinforced and VE-cadherin expression was affected, although to a lesser extent. We thus unmasked MARCH3 as a novel regulator of the endothelial barrier, as its silencing allows endothelial cells to resist to cytokine-triggered cortical actin remodeling and permeability increase. From a

Fig. 3. Tight junctions are strengthened in MARCH3-depleted cells. (A) Gene array analysis of siM3-transfected human brain endothelial cells compared to sic-transfected cells (Table S2). Heat-map of up- (>1.25, red) and down-regulated (<1.25, blue) genes, from siM3/sic fold changes in three independent experiments. The repartition of significantly (P < 0.05) up- and down-regulated sequences is shown (top diagram). Protein coding RNA (in yellow, 67% of total gene) were then classified according to their fold change in expression (siM3/sic, bottom diagram). (B, C) Table includes two of the best down- (blue) and up-regulated (red) genes (symbol names), together with P-value and fold change. Samples used for gene array were further processed for RT-PCR to validate main hits. (D, E) mRNA (D) and protein (E) expression of indicated targets were monitored 3 days post-transfection by RT-PCR and western-blots, respectively. Graphs showed quantification of fold change in three independent experiments. (F) Confocal analysis of tight junction-associated protein ZO-1 in sic- or siM3-transfected endothelial cells. Zoom on cell borders is shown on the right panel, together with an inverted image as processed by IMAGE J (FIJI). Scale bars: 10 μm. All panels are representative of at least three independent experiments. t-test *P < 0.05; **P < 0.01; ***P < 0.001.
molecular standpoint, our results are reminiscent of the signaling pathway operating downstream of AJ and regulating claudin-5 expression, where FoxO plays an instrumental role in steady state and/or recovery of the endothelial barrier integrity [9,13]. Indeed, MARCH3 silencing causes an up-regulation of both claudin-5 and occludin expression, which in turn might strengthen TJ architecture.

Further studies are required to identify MARCH3 molecular targets, as well as the type of ubiquitin modifications involved. Nevertheless, our study highlights the potential importance of ubiquitin ligases, particularly deubiquitylating enzymes, in modulating the composition of the endothelial junctions. Of note, it has been recently reported that occludin can be regulated by ubiquitin-targeted degradation [18,30] during the course of permeability increase. Interestingly, at least two different lysine residues on claudin have been reported to serve as ubiquitin chain acceptors [31,32]. This raised the possibility that MARCH3 might directly or indirectly modulate the ubiquitination/degradation status of tight junction protein complexes and requires further investigation.

In addition, the E3 ubiquitin ligase RNF152, which was found in our initial screen (Table S1), was recently acknowledged to direct the ubiquitylation of the RagA GTPase in response to amino acid starvation and thus to modulate mTORC1 lysosomal signaling [33]. Although we did not pursue on RNF152 in our study, it may be interesting to investigate the possible synergy between RNF152 and MARCH3 in regard to mTORC1 and lysosomal signaling in endothelial homeostasis and barrier integrity. There is now growing evidence that mTORC1 contributes to endothelial homeostasis [34], while FoxO1 emerged as an instrumental mediator of vascular remodeling operating at the interface between metabolic activity and quiescence/proliferation balance [12].

In conclusion, MARCH3 might sustain the basal activation of FoxO1, which can then be modulated to ensure a rapid and adaptive response of the endothelium. A better knowledge on the basic mechanisms involved in integrating microenvironmental cues and fundamental signaling pathways might improve our understanding of vascular dysfunctions, including exacerbated permeability that occurs in many diseases and human pathologies.

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**Fig. 4.** MARCH3 impact on OCLN gene expression relies on FoxO. (A, B) Human brain endothelial cells were transfected with siM3 or sic duplexes, processed for western-blots 3 days later, as indicated. (B) Alternatively, cells were stained for FoxO1 (green) and analyzed by confocal microscopy. Nuclei were counterstained with DAPI (blue). Graph showed the percentage of FoxO1-positive nuclei. Scale bars: 10 µm. n > 200 cells. t-test: *P < 0.05. (C) Cells were exposed to the FoxO1 inhibitor (AS1708727, iFoxo, 100 nM, 3 days) or vehicle (dmso) and processed for RT-PCR. (D) Permeability assays were performed in nonstimulated (ctl), histamine- (Hista) or IL-8-challenged HUVECs pretreated with dmso and iFoxo. All panels are representative of at least three independents experiments. ANOVA test *P < 0.05; ***P < 0.001.
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Author contributions
HML designed the research, conducted the experiments, and analyzed the data; GAG conducted the experiments and analyzed the data; LT designed the research, conducted the experiments, and wrote the manuscript. All authors have read and approved the manuscript.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. siRNA library screen.
Table S2. Gene array analysis.