Current Biology 19, 668–674, April 28, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.02.057

Report

VE-Cadherin-Mediated Cell-Cell Interaction Suppresses Sprouting via Signaling to MLC2 Phosphorylation

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Summary

During new blood vessel formation, the cessation of angiogenic sprouting is necessary for the generation of functional vasculature. How sprouting is halted is not known, but it is contemporaneous with the development of stable intercellular junctions [1]. We report that VE-cadherin, which is responsible for endothelial adherens junction organization [2, 3], plays a crucial role in the cessation of sprouting. Abrogating VE-cadherin function in an organotypic angiogenesis assay and in zebrafish embryos stimulates sprouting. We show that VE-cadherin signals to Rho-kinase-dependent myosin light-chain 2 phosphorylation, leading to actomyosin contractility [4], which regulates the distribution of VE-cadherin at cell-cell junctions. VE-cadherin antagonizes VEGFR2 signaling, and consequently, inhibition of VE-cadherin, Rho-kinase, or actomyosin contractility leads to VEGF-driven, Rac1-dependent sprouting. These findings suggest a novel mechanism by which cell-cell adhesion suppresses Rac1-dependent migration and sprouting by increasing actomyosin contractility at cell junctions.

Results

VE-Cadherin Suppresses Sprouting in an Organotypic Angiogenesis Assay and during Embryonic Development in Zebrafish

To investigate whether VE-cadherin-mediated homophilic interaction suppresses angiogenic sprouting, we first used VE-cadherin-blocking antibodies [5] in an organotypic assay. In this assay, capillary-like three-dimensional tubules arise from the interaction of human umbilical vein endothelial cells (HUVEC) with human dermal fibroblasts (HDF) [6, 7]. At 12–14 days, the tubules are largely quiescent [7] and show extensive accumulation of VE-cadherin at cell-cell junctions (Figure 1A). These tubules do not respond to VEGF stimulation by cell migration and sprouting (Figure S1A available online), whereas VEGF treatment in the earlier migratory phase increases angiogenesis (Figure S1A). Treatment with blocking antibody Cad5 at 50 μg/ml [5] resulted in rapid disassembly of established quiescent tubules, rounding, and endothelial cell death (Figure 1B) [2, 3]. However, treatment with Cad5 at 5 or 10 μg/ml resulted in induction of cell protrusions and sprouting (Figure 1B and Movies S1, S2, and S3; Figure S2A shows a schematic representation of treatments). Sprouting in response to 5 μg/ml Cad5 continued for ~36 hr (Figure 1B), followed by tubule disassembly, cell rounding, and death at 48 hr (Figure S2B). Treatment with 10 μg/ml Cad5 also resulted in protrusions and sprouting, but disassembly occurred within 12 hr (Figure 1B and Movie S3). Quantification of tubule formation following 5 μg/ml Cad5 treatment for 30 hr showed significant increases in the number of tubules, total tubule length, and branch points (Figure 1C). To rule out nonspecific effects of Cad5, we used blocking antibody BV9 that binds to a different part of the extracellular domain [5]. Treatment with 10 μg/ml BV9 resulted in increased tube formation (Figure S2C). VE-cadherin blockade delocalized VE-cadherin from cell junctions but did not affect its overall levels (Figure S2D). To determine whether these effects were from loss of VE-cadherin function or altered VE-cadherin signaling, we employed VE-cadherin siRNAs. VE-cadherin knockdown in HUVEC seeded on confluent fibroblasts [7] resulted in increased tubule formation, suggesting that it is loss of VE-cadherin that leads to increased angiogenesis (Figure S2E). These blocking antibody and siRNA experiments show that VE-cadherin-mediated cell-cell interaction suppresses sprouting and that VE-cadherin is not required for initial tubule assembly but is required for cell-cell adhesion and survival in established tubules.

To determine whether VE-cadherin suppresses sprouting in vivo, we used morpholino oligonucleotide-mediated knockdown of VE-cadherin expression in zebrafish embryos [8]. Figure 1D shows lateral views of the trunk region of embryos injected with VE-cadherin or control mismatch morpholino oligonucleotides at 30 hr postfertilization (hpf). Strikingly, the primary vascular network forms at the right place in VE-cadherin morphants; however, intersegmental vessels show increased sprouting (Figure 1D). Time lapse videos show that intersegmental vessels in control embryos get stabilized upon contact at the dorsal regions, whereas in VE-cadherin morphants, the vessels fail to connect and keep forming sprouts up to 46 hr hpf (Movies S4, S5, and S6). These data from in vivo and organotypic culture systems show that VE-cadherin-mediated adhesion suppresses sprouting during new vessel establishment. However, because recent work shows that VE-cadherin regulates junctional molecules such as claudin 5 [9], it is possible that other junctional components also contribute to suppression of sprouting.

VE-Cadherin Signals to Activate Rho-Kinase-Dependent MLC2 Phosphorylation at Intercellular Junctions

Sprouting in the migratory phase of cocultures requires down-regulation of Rho-kinase-dependent MLC2 phosphorylation [7]. Because sprouting is inhibited in the established phase, we investigated whether MLC2 phosphorylation is elevated.

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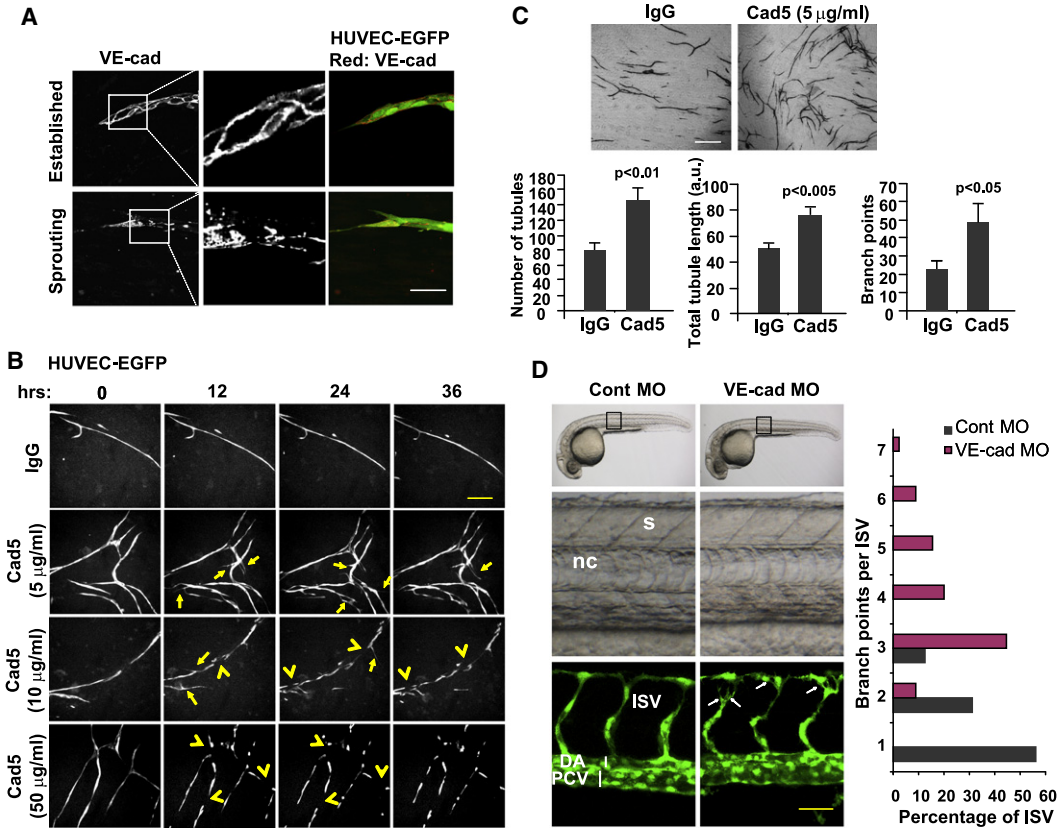

Figure 1. Partial VE-Cadherin Blockade in Organotypic Culture and VE-Cadherin Knockdown in Zebrafish Stimulate Sprouting

(A) VE-cadherin accumulation at junctions in established tubules. HUVEC-EGFP cocultured with HDF were fixed 7 days (migratory) or 12 days (established) after coculture and stained for VE-cadherin. Middle panels show magnifications of insets in left panels. Note the continuous distribution of VE-cadherin in established tubules. Scale bar represents 50 μm.

(B) Partial VE-cadherin blockade induces sprouting. Established tubules were treated with Cad5 (5 μg/ml, 10 μg/ml, or 50 μg) or control IgG and followed by time lapse microscopy. Video stills are at 12 hr intervals over 48 hr. Closed arrows show sprouting points; open arrows show areas of disassembly. Scale bar represents 50 μm.

(C) Partial VE-cadherin blockade increases tube formation. Established tubules treated with Cad5 (5 μg/ml) or control IgG for 30 hr were visualized by CD31 staining 3 days after the end of treatment. Number of tubules, total length, and number of branch points are represented as mean ± SEM (n = 9 microscopic fields from triplicate wells). Scale bar represents 100 μm.

(D) Increased sprouting of intersegmental vessels in zebrafish embryos with VE-cadherin knockdown. Shown is a lateral view of the trunk region of a representative control morpholino-injected transgenic fli1:EGFP zebrafish embryo (Cont-MO) or embryo injected with VE-cadherin morpholino oligonucleotide (VE-cad MO2) at 30 hpf. Upper panels show bright-field images of whole embryos showing normal development of VE-cad MO embryos. Middle panels show magnifications of top panel insets showing the notochord (nc) and somites (s). Lower panels show fluorescent images of trunk vasculature with three intersegmental vessels. Note the persistent sprouting (arrows) of intersegmental vessels (ISV) in VE-cadherin morphants. DA, dorsal aorta; PCV, posterior cardinal vein. Scale bar represents 50 μm. Histogram shows quantification of branching of ISVs.

Figure 2A shows that MLC2 phosphorylation is undetectable in sprouting tubules but is upregulated by Rho-kinase in the established tubules.

We investigated whether VE-cadherin signals to MLC2 phosphorylation. When HUVEC are cultured on matrigel, junctional accumulation of VE-cadherin can be seen at 48 hr and colocalizes with phosphorylated MLC2 (Figure 2B). A similar colocalization is seen in the established phase in the cocultures (Figure S4B). Treatment with 5 or 10 μg/ml Cad5 disrupted the organization of VE-cadherin and downregulated the phosphorylation of MLC2 at cell junctions without apparent effects on the level or localization of total MLC2 (Figures 2B and S3F; Figure S3A shows schematic representation of treatments). Immunoblotting confirmed that VE-cadherin blockade reduced phospho-MLC2 levels (Figures 2B and 2C). MLC2 phosphorylation was reduced by 42.0% and 45.2% with treatment with Cad5 at 10 μg/ml for 30 min and 60 min, respectively. With Cad5 at 5 μg/ml, there was 53.7% and 44.0% reduction in MLC2 phosphorylation at 4 hr and 24 hr, respectively. Treatment with BV9 also resulted in reduced MLC2 phosphorylation (Figure S3C). MLC2 phosphorylation is Rho-kinase-dependent, given that treatment with Rho-kinase inhibitor Y27632 [10] for 30 min and 60 min reduced phosphorylation by 91.6% and 84.3%, respectively, whereas treatment for 4 hr and 24 hr resulted in 92.1% and 73.3% reduction, respectively. Because Rho-kinase inhibition leads to increased cord formation in matrigel ([7] and Figure S3B), we investigated whether VE-cadherin blockade leads to increased cord formation. Treatment with Cad5 (5 μg/ml) increased cord formation (Figure 2C). Similarly, VE-cadherin knockdown by siRNA reduced MLC2 phosphorylation and increased cord formation (Figures 2D–2F and S3G).

Figure 2E shows that siRNAs against ROCKI and ROCKII reduced MLC2 phosphorylation and increased cord formation, confirming that the effects of Y27632 are through ROCKI and

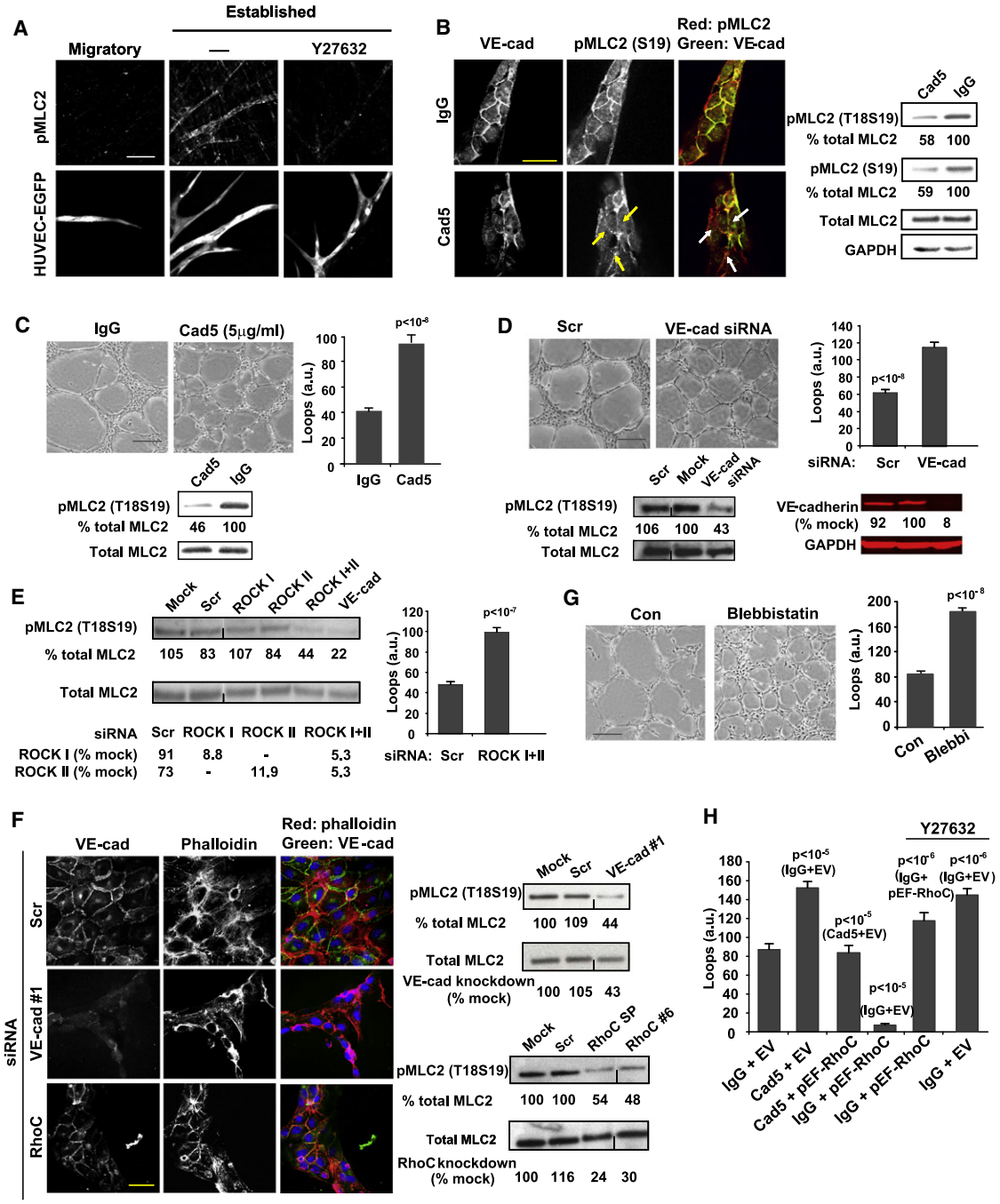

Figure 2. VE-Cadherin Regulates MLC2 Phosphorylation at Intercellular Junctions

(A) MLC2 phosphorylation is upregulated in established tubules. HUVEC-EGFP cocultured with HDF were fixed in migratory (7 days) or established (12 days) phases and stained for phospho-MLC2 (S19). Y27632 (10 μM) was applied for 5 hr before fixation. Scale bar represents 50 μm.

(B) VE-cadherin blockade delocalizes VE-cadherin and downregulates phosphorylated MLC2 at junctions. At 48 hr after plating on matrigel, HUVEC were treated with Cad5 (10 μg/ml) or control IgG for 15 min and stained for VE-cadherin and phospho-MLC2 (S19). Arrows show areas of concomitant VE-cadherin and phospho-MLC2 downregulation. Scale bar represents 50 μm. Western blot shows monophosphorylated (S19) and diphosphorylated (T18S19) MLC2 after Cad5 treatment (10 μg/ml) for 30 min.

(C) Partial VE-cadherin blockade increases cord formation. At 24 hr after plating on matrigel, HUVEC were treated with Cad5 (5 μg/ml) or control IgG for 24 hr. Loops counted at the end of treatment are represented as mean ± SEM (n = 9 microscopic fields). Scale bar represents 100 μm. Western blot shows levels of phospho-MLC2 (T18S19) after Cad5 treatment (5 μg/ml) for 4 hr.

(D–E) Knockdown of VE-cadherin or Rho-kinase I and Rho-kinase II downregulates MLC2 phosphorylation and increases cord formation in matrigel. HUVEC were transfected with siRNAs targeting VE-cadherin (D), Rho-kinase I and Rho-kinase II (E), or scrambled control. Loops counted 24 hr after plating on matrigel are represented as in (C). Scale bar represents 100 μm. Western blots show levels of phospho-MLC2 (T18S19) 24 hr after plating on matrigel.

(F) VE-cadherin or RhoC knockdown blocks stress fiber formation. HUVEC transfected with VE-cadherin, RhoC, or scrambled siRNAs in matrigel were stained for F-actin 48 hr after transfection. Nuclei were visualized by DAPI staining (blue). Scale bar represents 50 μm. Western blots show levels of phospho-MLC2 (T18S19).

ROCKII inhibition. Additionally, knockdown of p120 catenin resulted in decreased MLC2 phosphorylation and increased cord formation (Figure S3D); p120 catenin has been shown to regulate the levels of expression of VE-cadherin [11]. Because MLC2 phosphorylation controls actomyosin contractility, we tested whether direct inhibition of actomyosin contractility would increase cord formation. Treatment with blebbistatin [12] at concentrations that do not induce cell collapse [13] increased cord formation (Figures 2G and S3E). Overall, these data show that VE-cadherin signals via Rho-kinase activity to upregulate MLC2 phosphorylation and actomyosin contractility at endothelial cell junctions.

VE-cadherin signaling can activate RhoA [14]; however, we found that knockdown of RhoA does not affect tube formation or cord formation, and there was no reduction in RhoA activation when VE-cadherin was blocked (data not shown). However, silencing RhoC reduced MLC2 phosphorylation and stress fiber formation (Figure 2F), suggesting that VE-cadherin signals via RhoC to regulate Rho-kinase, MLC2 phosphorylation, and actomyosin contractility during tube formation. In support of this hypothesis, overexpression of RhoC reversed the increase in cord formation following VE-cadherin blockade (Figure 2H). RhoC overexpression alone decreased cord formation, and this could be reversed by Rho-kinase inhibitor Y27632.

Endothelial Cell Sprouting Requires Rac1 Activation

To determine whether MLC2 phosphorylation and actomyosin contractility in the established phase suppress sprouting, we used two structurally unrelated Rho-kinase inhibitors, Y27632 [10] and H1152 [15], or blebbistatin [12]. These treatments resulted in induction of sprouting (Figure 3A and Movies S7, S8, and S9; Figure S4A shows a schematic representation of treatments) and increased tube formation (Figures 3B and S4C). In contrast, HUVEC modified to overexpress MLC2 [16] showed decreased tube formation (41.5%; $p < 0.0005$). Together, these data show that VE-cadherin signaling to Rho-kinase and MLC2 phosphorylation suppresses sprouting.

Protrusive activity [17] has been associated with the activity of Rac1; therefore, we investigated whether partial VE-cadherin blockade leads to Rac1 activation. VE-cadherin blockade resulted in an increase of Rac1-GTP by 41% ± 10.0% ($n = 3$) (Figure S5A). Similarly, there was a 75.7% ± 18.4% ($n = 3$) increase in Rac1-GTP following Y27632 treatment (Figure S5B). Therefore, we asked whether Rac1 inhibition would inhibit sprouting. Treatment with NSC23766, an inhibitor of Rac1 activation [18], blocked the induction of sprouting in response to partial VE-cadherin blockade (Figure 3C and Movies S10 and S11) and the increase in tube formation (Figure 3D). Similarly, induction of sprouting and increased tube formation following inhibition of Rho-kinase or actomyosin contractility were blocked by inhibition of Rac1 activation (Figure 3E and Movies S12, S13, and S14). Whereas NSC23766 reduced Cdc42 activation at early time points, blockade of sprouting was seen at later time points when only Rac1 activation was reduced (Figure S5D). To confirm that the effects of NSC23766 were due to Rac1 inhibition in endothelial cells, we used siRNA knockdown of Rac1 in HUVEC prior to seeding on confluent fibroblasts. Figure 3F shows that Rac1 knockdown blocked the increase in tube formation in response to Rho-kinase inhibition.

Signaling from VE-Cadherin to MLC2 Phosphorylation Suppresses VEGF Receptor 2-Dependent Sprouting

Established tubules do not respond to VEGF (Figure S1); however, because VEGF receptor 2 (VEGFR2) signaling stimulates endothelial cell migration and activates Rac1 [19], we investigated whether VEGFR2 drives sprouting when VE-cadherin or Rho-kinase are inhibited. Increased angiogenesis in response to Rho-kinase inhibition or VE-cadherin blockade (Figure 3G) was reversed by blocking VEGF with Avastin [20] or by VEGFR2 inhibition with the selective inhibitor SU1498 [19] or BAY 43-9006 [21] (data not shown), as was the increase in Rac1-GTP in response to Rho-kinase inhibition (Figure S5B). Rho-kinase inhibition led to increased VEGFR2 phosphorylation (Figure S5C), arguing that suppression of sprouting by VE-cadherin signaling results from blocking VEGFR2 activation. Interestingly, we found that sprouting in Cad5-treated cocultures was not increased by adding VEGF (Figure S5E and Movies S15 and S16), suggesting that the VEGF produced by the fibroblasts in cocultures [7] is sufficient to fully activate the system.

Rho-Kinase-Dependent Actomyosin Contractility Regulates the Distribution of VE-Cadherin at Intercellular Junctions

Prolonged Rho-kinase inhibition deregulates VE-cadherin and enhances the permeability of endothelial monolayers [22]. Given our findings, we asked whether Rho-kinase and actomyosin contractility regulates VE-cadherin localization during tube formation. Figure 4A shows that Rho-kinase inhibition for 16 hr resulted in loss of the continuous distribution of VE-cadherin at intercellular junctions characteristic of quiescent tubules in the cocultures (Figure 1A). This altered distribution did not appear to reflect internalization of VE-cadherin into EEA1-positive vesicles [23] (data not shown). Total levels of VE-cadherin were unaffected when HUVEC were treated in matrigel with Y27632 (Figure S2D). Inhibition of actomyosin contractility with blebbistatin for 16 hr had a similar effect to Rho-kinase inhibition (Figure 4A). Less marked effects were observed within 4 hr of treatment (data not shown). These data show that, during establishment of newly formed vessels, VE-cadherin signaling through Rho-kinase and actomyosin contractility is required to maintain its uniform distribution along the endothelial adherens junction (Figures 1A and 4A).

Discussion

VE-cadherin is a major component of endothelial adherens junctions necessary for blood vessel integrity and endothelial cell survival [2, 3]. Our results show that VE-cadherin also suppresses Rac1-dependent vessel sprouting. The requirement for Rac1 in endothelial cell sprouting is consistent with the requirement of Rac1 for the generation of small branching vessels during vascular development [24]. Because sprouting is associated with increased vascular permeability, failure to

(G) Inhibition of actomyosin contractility increases cord formation. At 24 hr after plating on matrigel, HUVEC were treated with blebbistatin (50 μM) for 24 hr. Histogram shows number of loops as in (C). Scale bar represents 100 μm.

(H) RhoC expression rescues the effects of VE-cadherin blockade on cord formation. HUVEC transfected with pEF-RhoC or empty vector (EV) were plated on matrigel, and the cultures were treated with Cad5 (5 μg/ml) or control IgG for 24 hr. Histogram shows the number of loops as in (C). Scale bar represents 100 μm.

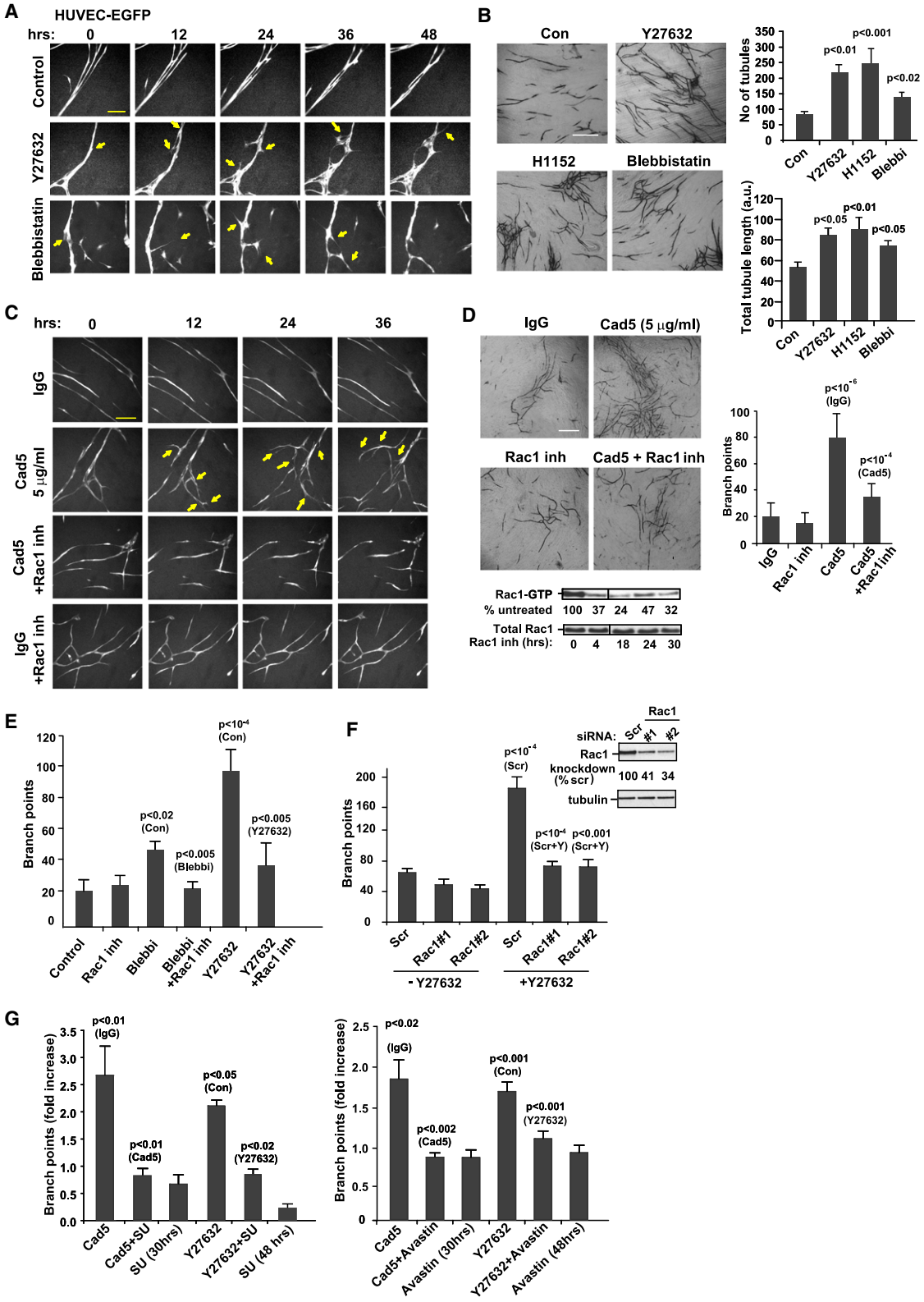

Figure 3. Inhibition of VE-Cadherin Signaling to MLC2 Phosphorylation Induces VEGFR2-Rac1-Dependent Sprouting

(A) Inhibition of Rho-kinase or actomyosin contractility stimulates sprouting. Established tubules treated with Y27632 (10 μm) or blebbistatin (5 μm) were followed by time lapse microscopy. Video stills are at 12 hr intervals over 48 hr. Arrows show sprouting points. Scale bar represents 50 μm.

(B) Inhibition of Rho-kinase or actomyosin contractility increases tubule formation. Established tubules treated with Y27632 (10 μM), H1152 (5 μm), or blebbistatin (5 μm) for 48 hr were visualized by CD31 staining 3 days after the end of treatment. The number of tubules and total tubule length are represented as mean ± SEM (n = 9 microscopic fields from triplicate wells). Scale bar represents 100 μm.

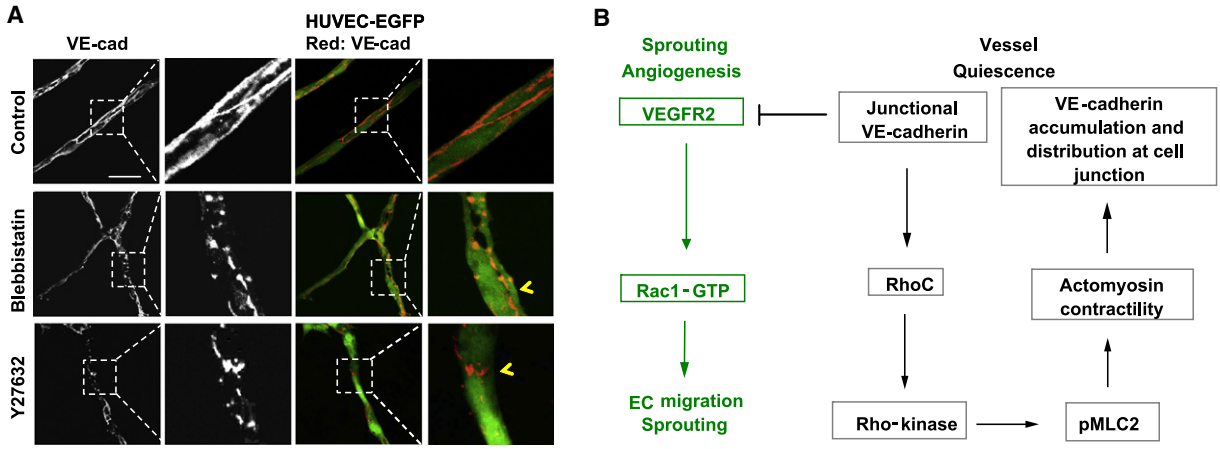

Figure 4. Rho-Kinase Inhibition Delocalizes VE-Cadherin from Intercellular Junctions

(A) Established tubules were treated with blebbistatin (5 μM) or Y27632 (10 μM) for 16 hr, fixed, and stained for VE-cadherin. In enlarged panels, note the loss of uniform distribution of VE-cadherin at cell junctions with inhibition of Rho-kinase or actomyosin contractility. Scale bar represents 50 μm.

(B) Model of interplay between VE-cadherin, Rho-kinase, phospho-MLC2, and actomyosin contractility acting to suppress the VEGFR2-Rac1-dependent sprouting.

suppress sprouting may contribute to the vascular remodelling defects and hemorrhages seen in VE-cadherin null embryos [3].

We show that, following tube formation in culture, VE-cadherin signals to increase actomyosin contractility via Rho-kinase. Signaling is likely to be via RhoC because knockdown of RhoC, but not RhoA, reduces MLC2 phosphorylation and stress fiber formation. This signaling pathway antagonizes VEGFR2 signaling to Rac1-dependent sprouting. Under conditions of homophilic adhesion, complexes between VE-cadherin and VEGFR2 have been shown to inhibit VEGFR2 phosphorylation and signaling to MAPK-ERK to suppress VEGF-driven proliferation [25]. Similarly, VE-cadherin may suppress VEGFR2 signaling to Rac1 to inhibit sprouting. Previously, endothelial cells from VE-cadherin null embryos were shown to have low levels of active Rac1 resulting from reduced junctional localization of the Rac1 exchange factor Tiam1 [26]. However, these studies were carried out in the absence of VEGF and would not have revealed activation of Rac1 through VEGFR2.

Studies in other systems show that VE-cadherin engagement at intercellular junctions signals to RhoA and Rac1 (reviewed in [27]). Inactivation of Rac1 in tissue culture results in defective endothelial cell migration, adhesion to substratum, and organization of intercellular junctions [24], suggesting that Rac1 has roles in both the angiogenic and quiescent states of the vasculature. Consistent with this notion, VE-cadherin signaling to Rac1 via Tiam1 stabilizes junctions [26]; indeed, we find in our system that blocking Rac1 activity destabilizes junctions (data not shown). However, activation of Rac1 by VEGF in endothelial monolayers can destabilize junctions by promoting internalization of VE-cadherin [23]. This indicates that the level and localization of Rac1 activation must be tightly regulated and suggests that there may be separate pools of activated Rac1 with different functions.

We show that following initial endothelial cell-cell assembly, VE-cadherin signaling to actomyosin contractility is necessary for the uniform distribution of VE-cadherin at cell junctions in the established quiescent state. Cavey and coworkers have shown that actomyosin contractility is required for even distribution of E-cadherin at epithelial cell junctions, arguing that contractility restricts the lateral mobility of junctional cadherins [28]. Interestingly, our data suggest that VE-cadherin, Rho-kinase, and actomyosin contractility act in a positive feedback loop because VE-cadherin signals to actomyosin contractility (Figure 4B). Such a positive feedback loop may be an advantageous mechanism for ensuring stable cell-cell junctions. Disruption of the loop at any point induces sprouting through reversing the suppression of VEGFR2 signaling to Rac1. Sprouting may also be suppressed at other points in the signaling network; for example, Rho-kinase could suppress Rac1 activation through activation of a Rac1 GTPase activating protein (GAP) [29].

(C) Established tubules treated with Cad5 (5 μg/ml) or control IgG in the presence or absence of Rac1 inhibitor NSC23766 (100 μM) were followed by time lapse microscopy. Video stills are at 12 hr intervals over 36 hr. Arrows show sprouting points.

(D) Increased tubule formation with partial VE-cadherin blockade is Rac1 dependent. Established tubules treated with 5 μg/ml Cad5 or control IgG for 30 hr in the presence or absence of the Rac1 inhibitor NSC23766 (100 μM) were visualized by CD31 staining 3 days after the end of treatment. Branch points are represented as in (B). Scale bar represents 100 μm. Western blot shows Rac1-GTP after Rac1 inhibitor treatment in matrigel. Quantification shows levels as percentage of Rac1-GTP compared to untreated controls after normalization for total Rac1.

(E) Increased tubule formation following inhibition of Rho-kinase or actomyosin contractility is Rac1 dependent. Established tubules treated with Y27632 (10 μM) or blebbistatin (5 μM) for 48 hr in the presence or absence of the Rac1 inhibitor NSC23766 (100 μM) were visualized by CD31 staining 3 days after end of treatment. Branch points are represented as in (B). Scale bar represents 100 μm.

(F) Increased tubule formation with Rho-kinase inhibition is reversed by Rac1 siRNA. HUVEC transfected with Rac1 siRNAs were plated on confluent fibroblasts. Tubule formation was visualized by CD31 staining after 5 days, and branch points are represented as in (B). Scale bar represents 100 μm. Western blot shows Rac1 expression levels.

(G) Increased branching with partial VE-cadherin blockade or Rho-kinase inhibition is reversed by VEGFR2 inhibition. Established tubules treated with Cad5 or Y27632 alone as in (D) and (E) or in combination with SU1498 (2.5 μM) or Avastin (5 μg/ml) were visualized by CD31 staining. Histograms show fold increase in branch points compared to controls.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, 6 figures, and 16 movies and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00810-0.

Acknowledgments

We thank R. Marais for the Bay 43-9006 inhibitor; A. Randi and TCS CellWorks for discussion; and C. Isacke for commenting on the manuscript. We thank Fredrik Wallberg for FACS sorting. C.J.M. is a Gibb Life Fellow of Cancer Research UK. Work in C.J.M.'s laboratory is supported by Cancer Research UK.

Received: May 8, 2008
Revised: February 1, 2009
Accepted: February 20, 2009
Published online: April 2, 2009

References

1. Dejana, E. (2004). Endothelial cell-cell junctions: Happy together. Nat. Rev. Mol. Cell Biol. *5*, 261–270.
2. Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., Lampugnani, M.G., Martin-Padura, I., Stoppacciaro, A., Ruco, L., et al. (1999). Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. Proc. Natl. Acad. Sci. USA *96*, 9815–9820.
3. Carmeliet, P., Lampugnani, M.G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oostuyse, B., Dewerchin, M., et al. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell *98*, 147–157.
4. Ikebe, M., and Hartshorne, D.J. (1985). Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. J. Biol. Chem. *260*, 10027–10031.
5. Corada, M., Liao, F., Lindgren, M., Lampugnani, M.G., Breviario, F., Frank, R., Muller, W.A., Hicklin, D.J., Bohlen, P., and Dejana, E. (2001). Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood *97*, 1679–1684.
6. Bishop, E.T., Bell, G.T., Bloor, S., Broom, I.J., Hendry, N.F., and Wheatley, D.N. (1999). An in vitro model of angiogenesis: Basic features. Angiogenesis *3*, 335–344.
7. Mavria, G., Vercoulen, Y., Yeo, M., Paterson, H., Karasarides, M., Marais, R., Bird, D., and Marshall, C.J. (2006). ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. Cancer Cell *9*, 33–44.
8. Lawson, N.D., and Weinstein, B.M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev. Biol. *248*, 307–318.
9. Taddei, A., Giampietro, C., Conti, A., Orsenigo, F., Breviario, F., Pirazzoli, V., Potente, M., Daly, C., Dimmeler, S., and Dejana, E. (2008). Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat. Cell Biol. *10*, 923–934.
10. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., et al. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature *389*, 990–994.
11. Xiao, K., Allison, D.F., Buckley, K.M., Kottke, M.D., Vincent, P.A., Faundez, V., and Kowalczyk, A.P. (2003). Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. J. Cell Biol. *163*, 535–545.
12. Straight, A.F., Cheung, A., Limouze, J., Chen, I., Westwood, N.J., Sellers, J.R., and Mitchison, T.J. (2003). Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science *299*, 1743–1747.
13. Wilkinson, S., Paterson, H.F., and Marshall, C.J. (2005). Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. Nat. Cell Biol. *7*, 255–261.
14. Nelson, C.M., Pirone, D.M., Tan, J.L., and Chen, C.S. (2004). Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. Mol. Biol. Cell *15*, 2943–2953.
15. Ikenoya, M., Hidaka, H., Hosoya, T., Suzuki, M., Yamamoto, N., and Sasaki, Y. (2002). Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. J. Neurochem. *81*, 9–16.
16. Pinner, S., and Sahai, E. (2008). PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. Nat. Cell Biol. *10*, 127–137.
17. Aspenstrom, P., Fransson, A., and Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. Biochem. J. *377*, 327–337.
18. Gao, Y., Dickerson, J.B., Guo, F., Zheng, J., and Zheng, Y. (2004). Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc. Natl. Acad. Sci. USA *101*, 7618–7623.
19. Garrett, T.A., Van Buul, J.D., and Burridge, K. (2007). VEGF-induced Rac1 activation in endothelial cells is regulated by the guanine nucleotide exchange factor Vav2. Exp. Cell Res. *313*, 3285–3297.
20. Wang, Y., Fei, D., Vanderlaan, M., and Song, A. (2004). Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. Angiogenesis *7*, 335–345.
21. Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M., et al. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. *64*, 7099–7109.
22. van Nieuw Amerongen, G.P., Beckers, C.M., Achekar, I.D., Zeeman, S., Musters, R.J., and van Hinsbergh, V.W. (2007). Involvement of Rho kinase in endothelial barrier maintenance. Arterioscler. Thromb. Vasc. Biol. *27*, 2332–2339.
23. Gavard, J., and Gutkind, J.S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat. Cell Biol. *8*, 1223–1234.
24. Tan, W., Palmby, T.R., Gavard, J., Amornphimoltham, P., Zheng, Y., and Gutkind, J.S. (2008). An essential role for Rac1 in endothelial cell function and vascular development. FASEB J. *22*, 1829–1838.
25. Grazia Lampugnani, M., Zanetti, A., Corada, M., Takahashi, T., Balconi, G., Breviario, F., Orsenigo, F., Cattelino, A., Kemler, R., Daniel, T.O., et al. (2003). Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148. J. Cell Biol. *161*, 793–804.
26. Lampugnani, M.G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V., and Dejana, E. (2002). VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. Mol. Biol. Cell *13*, 1175–1189.
27. Liebner, S., Cavallaro, U., and Dejana, E. (2006). The multiple languages of endothelial cell-to-cell communication. Arterioscler. Thromb. Vasc. Biol. *26*, 1431–1438.
28. Cavey, M., Rauzi, M., Lenne, P.F., and Lecuit, T. (2008). A two-tiered mechanism for stabilization and immobilization of E-cadherin. Nature *453*, 751–756.
29. Ohta, Y., Hartwig, J.H., and Stossel, T.P. (2006). FilGAP, a Rho- and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling. Nat. Cell Biol. *8*, 803–814.