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RacGAP1-driven focal adhesion formation promotes melanoma transendothelial migration through mediating adherens junction disassembly

Pu Zhang [a, b, *], Huiyuan Bai [a], Changliang Fu [b], Feng Chen [a], Panying Zeng [a], Chengxiang Wu [a], Qichao Ye [a], Cheng Dong [b], Yang Song [a], Erqun Song [a]

[a] College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, China
[b] Department of Bioengineering, Pennsylvania State University, University Park, PA 16801, USA

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ABSTRACT

Melanoma cell migration across vascular endothelial cells is an essential step of tumor metastasis. Here, we provide evidence that RacGAP1, a cytokinesis-related Rho GTPase-activating protein, contributed to this process. Depletion of RacGAP1 with RacGAP1-targeting siRNA or overexpression of RacGAP1 mutant (T249A) attenuated melanoma cell transendothelial migration and concomitant changes of adherens junctions. In addition, RacGAP1 promoted the activations of RhoA, FAK, paxillin and triggered focal adhesion formation and cytoskeletal rearrangement. By overexpressing FAK-related non-kinase (FRNK) in endothelium, we showed that RacGAP1 mediated endothelial barrier function loss and melanoma transmigration in a focal adhesion-dependent manner. These results suggest that endothelial RacGAP1 may play critical roles in pathogenic processes of cancer by regulating endothelial permeability.

1. Introduction

Melanoma metastasis is a highly regulated process requiring tumor adhesion to and migration cross the endothelial cells lining the blood vessel [1–5]. When tumor cells undergo extravasation, they trigger endothelial junction breakdown [6,7]. The integrity of endothelial junction is maintained by the balance between cell–cell adhesive interactions and cell contractility [8,9]. Vascular endothelial (VE)-cadherin homophilic bindings maintain endothelial cell–cell interactions, sealing the adherens junctions [10,11]. At resting states, VE-cadherin is localized to the adherens junctions, associating with α-catenin, β-catenin, p120-catenin and plakoglobin via its cytoplasmic domains. Upon stimulation, the phosphorylation of VE-cadherin tyrosine residues results in the dissociation of p120 and β-catenin complex from cytoplasmic tails of VE-cadherin, which in turn disrupts endothelial junction integrity [12,13]. The dissociation of VE-cadherin and β-catenin is necessary for VE-cadherin internalization and elevated endothelium permeability [14]. Cytoskeletal rearrangement, such as cortical actin dissolution and actin stress fiber formation, and actomyosin machinery-mediated cell contractility also contributes to the events of endothelial retraction and gap formation [15–18].

Two RhoGTPases, RhoA and Rac, has been implicated in the process of endothelial junction breakdown [19]. RhoA induces junction breakdown by controlling actomyosin contractility, while Rac stabilizes endothelial junction and antagonizes the activity of RhoA [20]. The cycling of RhoGTPases between active and inactive states is controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which can determine the GTP-bound states of RhoA and Rac. One of RhoGTPase GAPs, RacGAP1 (also known as MgcRacGAP or hCYK-4), is a component of the centralspindlin complex, mediating Rho-dependent signaling required for the actomyosin contractile ring assembly during cytokinesis [21,22]. It also plays important roles in controlling cell proliferation and differentiation of hematopoietic cells. Recently, RacGAP1 was found to mediate Rac to RhoA activation switch [23,24]. Phosphorylated RacGAP1 is recruited to invasive pseudopods, locally suppressing the activity of the cytoskeletal regulator

Abbreviations: FAK, focal adhesion kinase; HUVEC, human umbilical vein endothelial cell; HMVEC, human microvascular endothelial cell; FRANK, FAK-related non-kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; TER, transendothelial electrical resistance; WT, wildtype; VE-cadherin, vascular endothelial-cadherin.

* Corresponding author. College of Pharmaceutical Sciences, Southwest University, Beibei, Chongqing 400715, China. Fax: +86 23 68251225.
E-mail addresses: pxz122@swu.edu.cn, pxz122@gmail.com (P. Zhang).

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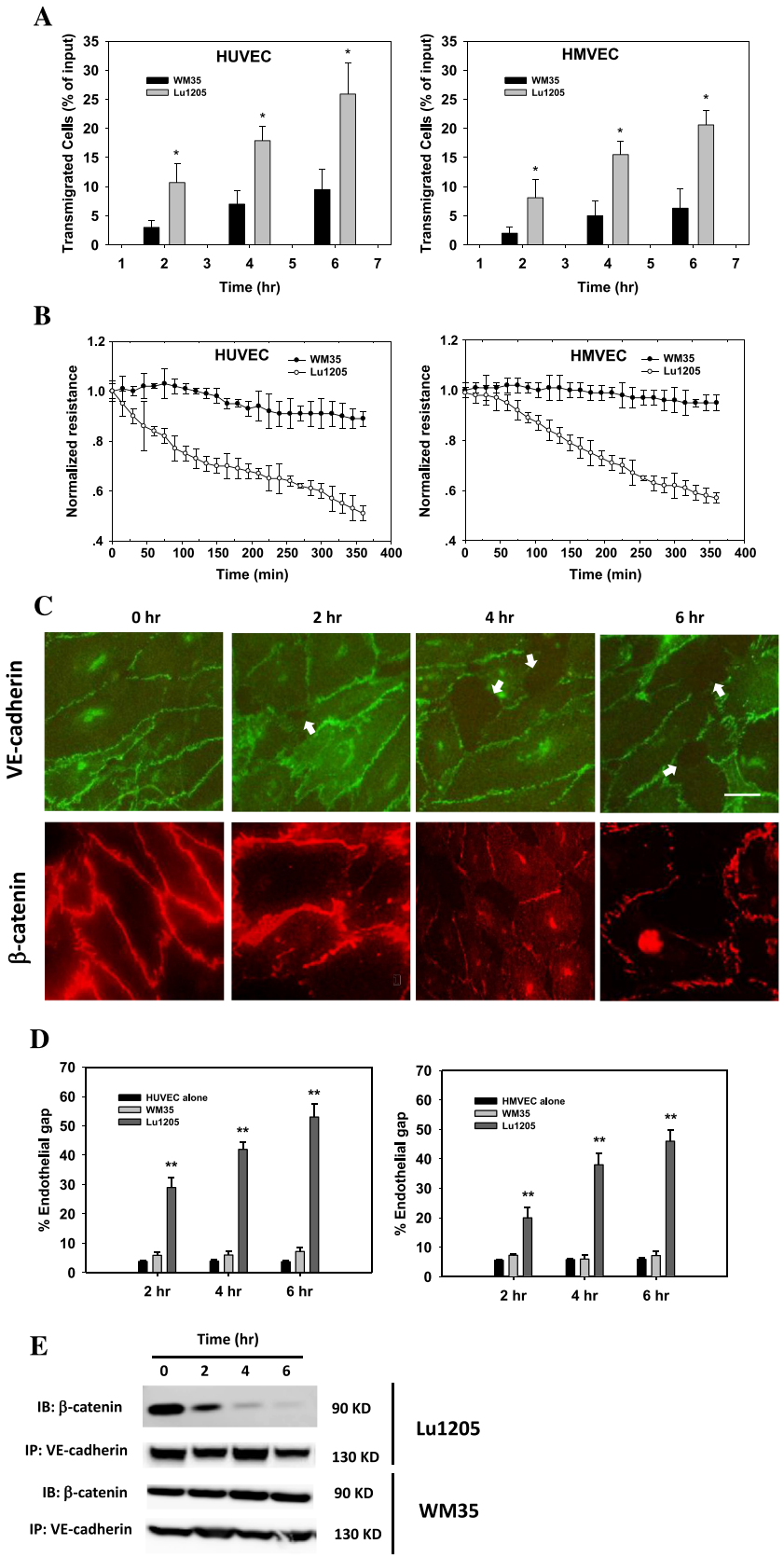

Fig. 1. Melanoma cell transendothelial migration and its effect on endothelial permeability. WM35 and Lu1205 cells were loaded on HUVEC or HMVEC monolayer in transwell insert and incubated for a variety of time durations, then the cells that had migrated to the lower chamber (A) and the temporal changes of TER (B) were measured. The changes of distributions of VE-cadherin and β-catenin in HUVECs co-cultured with Lu1205 cells were detected with immunofluorescence. Arrow: gap formation. Bar: 10 μm (C). Percentage of endothelial gap formation induced by co-culturing HUVECs or HMVECs with Lu1205 or WM35 cells were quantified (D). Time-dependent dissociation of VE-cadherin and β-catenin in HUVECs or HMVECs co-cultured with Lu1205 and WM35 cells was detected with immunoprecipitation (E). Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with WM35.

Rac and promoting the activation of RhoA in this subcellular region. However, it is unknown whether RacGAP1 plays any roles in mediating endothelial junction breakdown and melanoma metastasis.

In the current study, we investigated signaling transductions involved in melanoma cell transendothelial migration. We found that RacGAP1 signaling in HUVECs and human microvascular endothelial cells (HMVECs) was critical for Lu1205 melanoma extravasation. RacGAP1 mediated activations of RhoA, FAK and paxillin and stress fiber formation, thereby inducing loss of endothelial junction integrity.

2. Materials and methods

2.1. Cell culture

human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in F12-K medium with 10% FBS, 30 μg/ml of endothelial cell growth supplement, 50 μg/ml heparin (Mallinckrodt Baker), and 100 U/ml of penicillin–streptomycin (Biofluids). HMVECs were obtained from Clonetics (San Diego, CA) and cultured in EGM-MV medium, containing microvascular endothelial cell growth factors and 10% FBS. The Lu1205 melanoma cell line (obtained from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% FBS and 100 U/ml of penicillin–streptomycin. WM35 melanoma cells (provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) were maintained in Roswell Memorial Park Institute (RPMI) supplemented with 10% FBS and 100 U/ml of penicillin–streptomycin. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.2. Plasmid transfection

A codon-optimized FLAG-RacGAP1^{WT} was synthesized by GenScript (Nanjing, China) and cloned into the pcDNA3.1 vector at

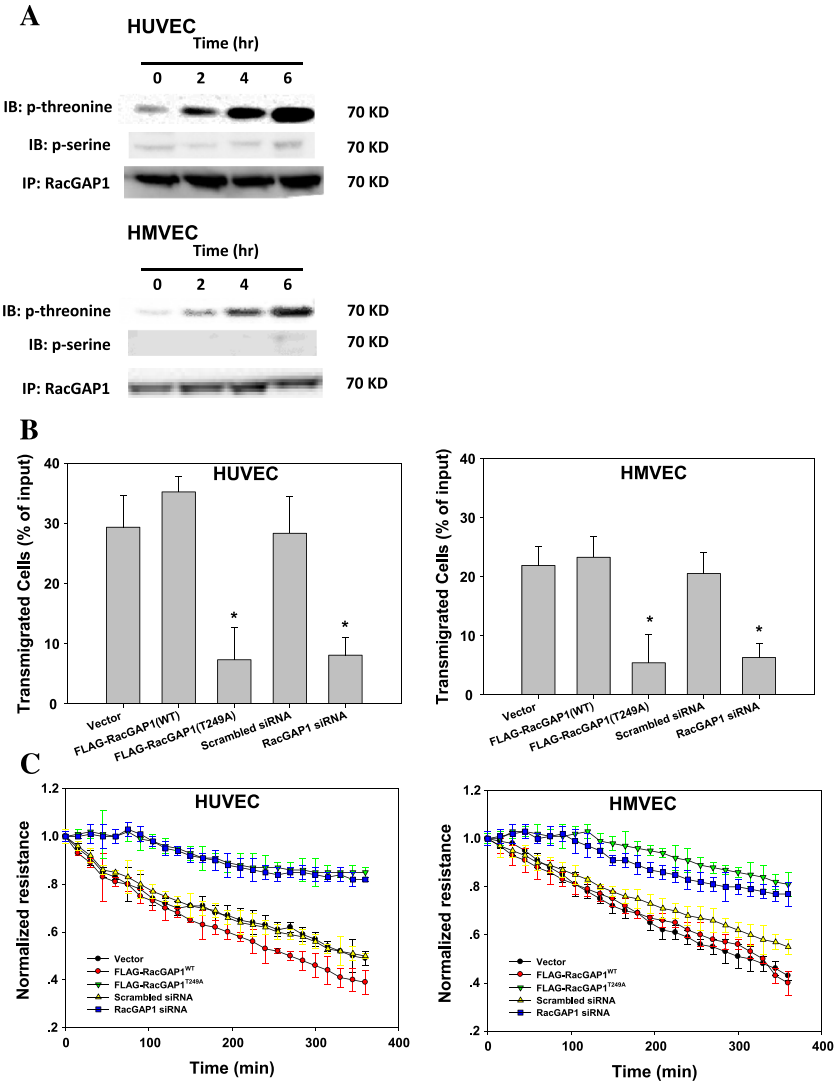

Fig. 2. RacGAP1 was involved in melanoma transendothelial migration and endothelial permeability. (A) Time-dependent RacGAP1 phosphorylation in HUVECs or HMVECs during Lu1205 transendothelial migration was measured with immunoprecipitation. RacGAP1 was immunoprecipitated with anti-RacGAP1 and detected with anti-phospho-serine and anti-phospho-threonine antibodies. Effects of transfections of vector, FLAG-RacGAP^{WT}, FLAG-RacGAP1^{T249A}, scrambled siRNA and RacGAP1 siRNA on Lu1205 cell transendothelial migration (B) and the temporal changes of TER (C) were determined. The changes of distributions of VE-cadherin and β-catenin in HUVECs which received vector, FLAG-RacGAP^{WT}, FLAG-RacGAP1^{T249A}, scrambled siRNA or RacGAP1 siRNA in co-culture with Lu1205 cells were detected with immunofluorescence. Arrow: gap formation. Bar: 10 μm. (D) Effects of transfections of vector, FLAG-RacGAP^{WT}, or FLAG-RacGAP1^{T249A} on the dissociation of VE-cadherin and β-catenin in HUVECs or HMVECs co-cultured with Lu1205 (E). Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ compared with control.

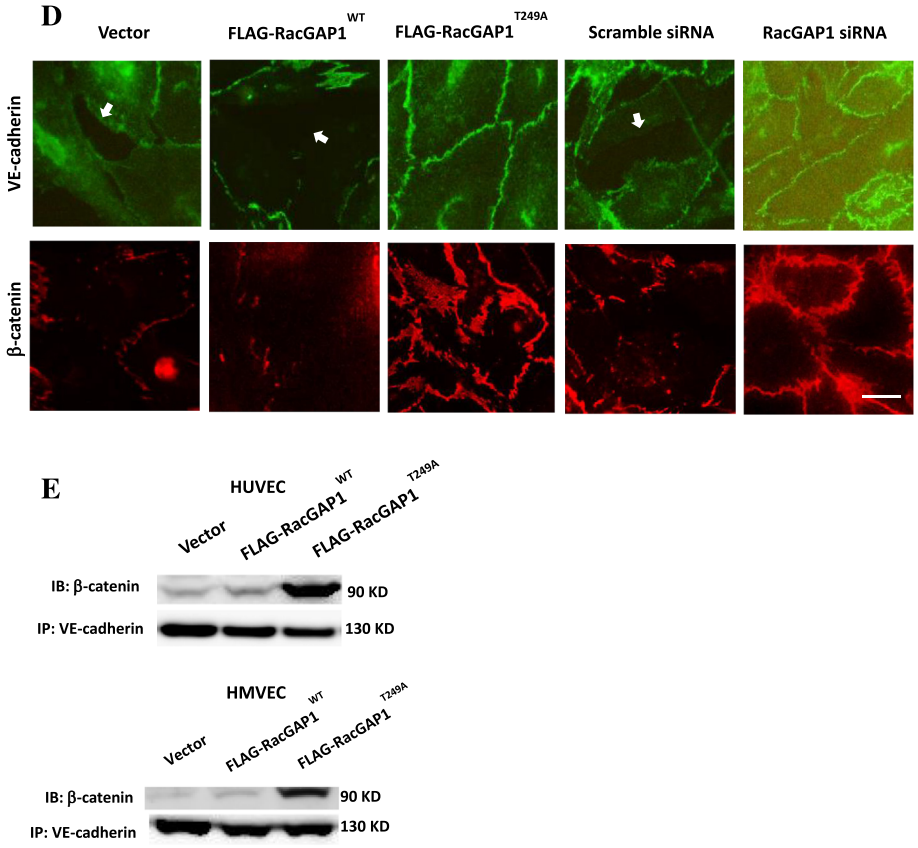

Fig. 2. (*continued*).

BamHI/EcoRI digestion sites. To generate FLAG-RacGAP1^{T249A}, the point mutation (T249A) was introduced by QuickChange II site-directed mutagenesis kit (Agilent Technologies, Palo Alto, CA) with primer 5′-AGCCGGCGGAAGGCAGGCACTCTGC-3′. FRNK construct was amplified from a pRKvsv-FRNK plasmid (generously provided by Dr. Kenneth Yamada, National Institute of Health, Bethesda, MD) by using primers: 5′-TCCGGATCCATGGAATCCA-GAAGACAGGCTAC-3′ and 5′-CCGGAATTCTCAGTGTGGCCGTGTC TGCCCTA-3′. The products were cloned into pcDNA3.1 with BamHI/EcoRI sites. Empty pcDNA3.1 plasmid was employed as negative control. HUVECs and HMVECs were transfected with 2.5 μg plasmids complexed in 7.5 μg Mirus TransIT 2020. The transfected cells were subjected to G418 selection for 2–3 weeks. Expression of the transfected constructs was assessed by Western blot analysis.

2.3. siRNA transfection

ON-TARGETplus scrambled siRNA and SMARTpool RacGAP1 siRNA were purchased from Thermo Scientific (Rockford, IL). HUVECs and HMVECs were plated in a 6-well plate and were 50–70% confluent when siRNA was introduced. Transfection was performed with 50 nM siRNA complexed with 7.5 ml RNAiMax transfection reagent (Thermo Scientific). Medium was changed after 6 h. The transfection efficiency to knockdown RacGAP1 reached 80% after 48 h as assessed by Western blotting.

2.4. Western blotting

HUVECs or HMVECs were collected and rinsed with PBS, and lysed with RIPA lysis buffer (20 mM Tris, 5 mM $MgCl_2$, 1 mM PMSF, 20 mg/ml aproptonin, 10 mg/ml leupeptin, 1 mM Na_3VO_3, and 20 mM β-glycerophosphate). The lysates were centrifuged at 14,000 rpm for 15 min. The protein concentrations across samples were checked by Bradford method. The samples were denatured by adding SDS running buffer (0.2% bromophenol blue, 4% SDS, 100 mM Tris [pH 6.8], and 20% glycerol) and β-mercaptoethanol. The samples were analyzed by SDS–PAGE on 12% gels. After the proteins were transferred to nitrocellulose membrane, FAK (Abcam, Eugene, OR), pY397 FAK (Abcam, Eugene, OR), paxillin (Santa Cruz, Dallas, TX), pY118 paxillin (Cell Signaling Technology, Danvers, MA), RhoA (Abcam, Eugene, OR), VE-cadherin (Cell Signaling Technology, Danvers, MA) , β-catenin (Cell Signaling Technology, Danvers, MA), RacGAP1(Santa Cruz, Dallas, TX), phosphor-serine (Abcam, Eugene, OR) were detected with corresponding primary monoclonal antibodies followed by HRP conjugated secondary antibodies. The labeled proteins were visualized using a chemiluminescence kit.

2.5. Immunoprecipitation (IP)

HUVEC lysates (1–2 mg of protein/ml) were mixed with appropriate IP antibodies at 4 °C for 12 h.

The Ag–Ab complexes were incubated with protein A conjugated agarose beads for 4 h at 4 °C. The antibody complex was obtained by centrifugation at 5000*g* for 5 min and the pellet was washed in ice-cold PBS. The Ag–Ab complexes were dissociated from agarose beads in SDS loading buffer. Immunoblotting (IB) was performed to detect the relevant immunoprecipitated proteins.

2.6. Immunofluorescence

HUVECs were grown on cover slips coated with fibronectin (1 μg/ml) before being co-cultured with melanoma cells. HUVECs

were then washed with PBS and fixed with 5% paraformaldehyde for 10 min. Cells were permeabilized with 0.3% Triton X-100 in PBS and blocked for 30 min with 5% BSA. Subsequently, cover slips were incubated with anti-VE-cadherin, anti-β-catenin or anti-paxillin for 1 h at room temperature. This was followed by staining with Alexa 555 or Alexa 488-conjugated anti-rabbit IgG. To image actin filaments, rhodamine-phalloidin (1:40; Life Technologies, Carlsbad, CA) was incubated with cells. Finally, fluorescent staining was visualized with IX71 Olympus inverted microscopy (Olympus, Inc.) with 40× magnification. Colocalization of paxillin and actin was processed by Image J. To analyze the size and number of paxillin-containing focal adhesions, images were background subtracted before thresholding and segmentation were conducted to detect the edges of focal adhesions. Then, the mean size (in pixels) and number of focal adhesions in each cell were calculated.

Disruption of VE-cadherin was identified from analysis of discontinuity of green fluorescence at VE-cadherin junctions between HUVECs. Gap area within disrupted VE-cadherin junctions was determined from six images. Gap area was quantified as the ratio of pixels within all the gaps to the total number of pixels of an image.

2.7. Transendothelial migration assay

HUVECs and HMVECs were grown on the filter membrane of transwell inserts (Corning, NY) to form monolayer. Thereafter, equal numbers (2.5×10^5) of melanoma cells were plated in the inserts. Inserts were placed in corresponding wells of a 24-well plate with 10% FBS in the bottom wells. The cells were allowed to transmigrate across the porous membrane filter. Endothelial cells and unmigrated cells at the top of the membrane filter were removed by a cotton swab while the cells at the bottom of the filter were fixed in 5% formaldehyde, and stained using calcein AM (Molecular Probes, Eugene, OR). The number of transmigrated cells were counted and normalized against total number of cells seeded.

2.8. Transendothelial electrical resistance (TER)

TER with respect to time was measured with Millicell ERS-2 Voltohmmeter (Millipore, Billerica, MA). The final TER values were calculated as ohm cm^2 by multiplying it with surface area of transwell insert. The results were presented as a percentage compared to normal HUVECs or HMVECs without melanoma cell co-cultured.

2.9. Rho activation assay

Rho activation assay kit (Upstate Biotechnology, Lake Placid, NY) was employed to determine the activity of RhoA in HUVECs and HMVECs being co-cultured with melanoma cells. Briefly, cell lysates were incubated with rhotekin Rho binding peptide coated on agarose beads for 1 h, and the GTP-Rho bound to rhotekin-agarose beads were immunoprecipitated and subject to Western blotting detection with anti-RhoA mouse IgG antibody (Santa Cruz).

2.10. Statistics

Statistical significance of differences between means was determined by using a Student's *t*-test or analysis of variance (ANOVA). Turkey's test was used for *post hoc* analysis for ANOVA. Probability values of $p < 0.05$ and $p < 0.01$ were chosen as statistical significant.

3. Results

3.1. Melanoma transendothelial migration induced adherens junction disassembly in HUVECs and HMVECs

To determine whether melanoma metastatic potentials were correlated with their transendothelial migration capacities, we used transwell migration assay. Time-dependent increase of transmigrated metastatic Lu1205 melanoma cells was observed (Fig. 1A). In the meanwhile, Lu1205 cell transmigration induced a time-dependent reduction of TER (Fig. 1B). In sharp contrast, non-metastatic WM35 cells did not migrate efficiently and failed to enhance endothelial permeability during the assay time course (Fig. 1A and B). Since increased endothelial permeability is usually associated with loss of adherens junction integrity, we detected the changes of junction proteins, VE-cadherin and β-catenin, in the

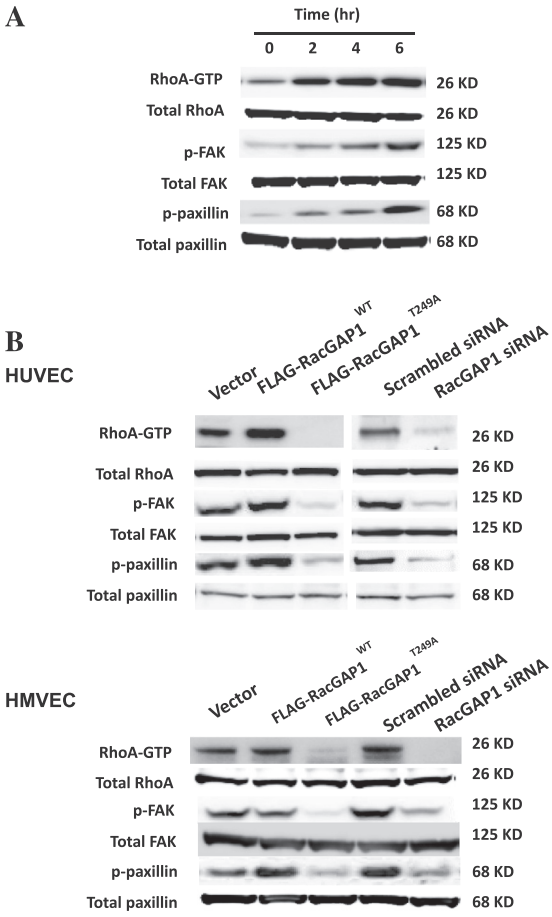

Fig. 3. RacGAP1 mediated activations of RhoA, paxillin and FAK and promoted focal adhesion formation during melanoma transendothelial migration. (A) Confluent HUVECs were co-cultured with Lu1205 cells for indicated time periods before being subject to Western blotting analysis of GTP-coupled RhoA, Y397 phosphorylated FAK, and Y118 phosphorylated paxillin. Total RhoA, FAK and paxillin serve as loading controls. (B) The activities of RhoA, FAK and paxillin in mutant construct- and siRNA-transfected HUVECs or HMVECs co-cultured with Lu1205 were measured with Western blotting. (C) Different HUVEC transfectants were co-cultured with Lu1205 cells for 6 h. HUVECs were stained with rhodamine-phalloidin and paxillin antibody. The right panel shows magnified views of the boxed area in the merged images. Bar = 10 μm. (Geen = paxillin, red = F-actin). (D) Quantification of the average number and size (μm^2) of paxillin-containing focal adhesions in HUVECs or HMVECs using ImageJ software. 12 cells were analyzed per condition in each experiment. Data were expressed as mean ± SEM of three independent experiments. $^*p < 0.05$ compared with control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

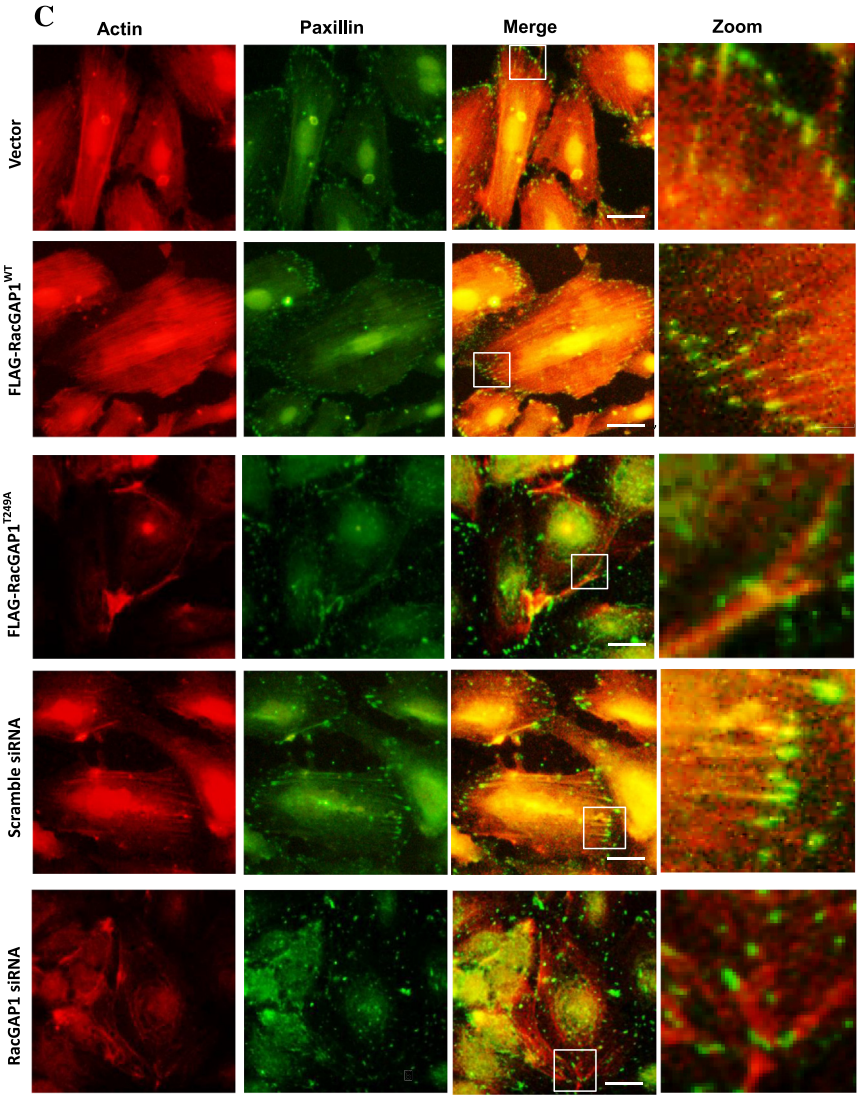

Fig. 3. (*continued*).

process. Intact HUVECs exhibited bright immunostaining of VE-cadherin and β-catenin at cell–cell junctions. Upon HUVEC-Lu1205 co-culture, endothelial VE-cadherin and β-catenin staining became discontinuous and segmented (Fig. 1C). 1×10^6 Lu1205 but not WM35 in direct contact with HUVEC or HMVEC monolayer induced endothelial gap formation in a time-dependent manner (Fig. 1D). HUVEC and HMVEC gap size were increased by 13-fold and 8-fold upon co-culturing endothelial monolayer and Lu1205 for 6 h. Concomitantly, Lu1205 contact induced a dissociation of β-catenin from VE-cadherin (Fig. 1E).

3.2. RacGAP1 plays an essential role in coordinating endothelial junction breakdown and melanoma migration

RacGAP1, a regulator of cytokinesis, was recently found to be recruited to cell adhesion sites and cell–cell junctions, regulating cellular functions [23,25]. Therefore, we hypothesized that RacGAP1 might play roles in regulating melanoma-mediated junction breakdown. By using immunoprecipitation, we demonstrated that RacGAP1 was phosphorylated in HUVECs and HMVECs co-cultured with Lu1205 cells in a time-dependent manner (Fig. 2A). To investigate whether RacGAP1 might be associated with Lu1205 cell transendothelial migration, the effects of different RacGAP1 constructs or siRNA silencing were tested. While a RacGAP1 wildtype construct (FLAG-RacGAP1^{WT}) slightly increased tumor migration, a mutant RacGAP1 carrying a point mutations of threonine 249 to alanine (FLAG-RacGAP1^{T249A}) significantly attenuated the migration of melanoma cells (Fig. 2B). To further verify the results of mutant construct transfection experiments, we detected cell responses when RacGAP1 was silenced with siRNA. siRNA-mediated knockdown of RacGAP1 remarkably reduced Lu1205 transmigration potencies compared with scrambled siRNA control. The reduction in Lu1205 migration across FLAG-RacGAP1^{T249A}-transfected or RacGAP1-depleted HUVECs or HMVECs observed was accompanied with elevation of TER compared with controls (Fig. 2C). In addition, Lu1205 adhesion-induced disruptions of VE-cadherin and β-catenin staining were abolished in HUVECs overexpressing FLAG-RacGAP1^{T249A} and being depleted of RacGAP1 by siRNA (Fig. 2D). Overexpressing FLAG-RacGAP1^{WT} caused a dissociation of VE-cadherin and β-catenin, while overexpressing FLAG-RacGAP1^{T249A} resulted in re-association of VE-cadherin and β-catenin, as assessed by immunoprecipitation (Fig. 2E). These results strongly supported that Lu1205 melanoma extravasation was dependent on RacGAP1-initiated pathway.

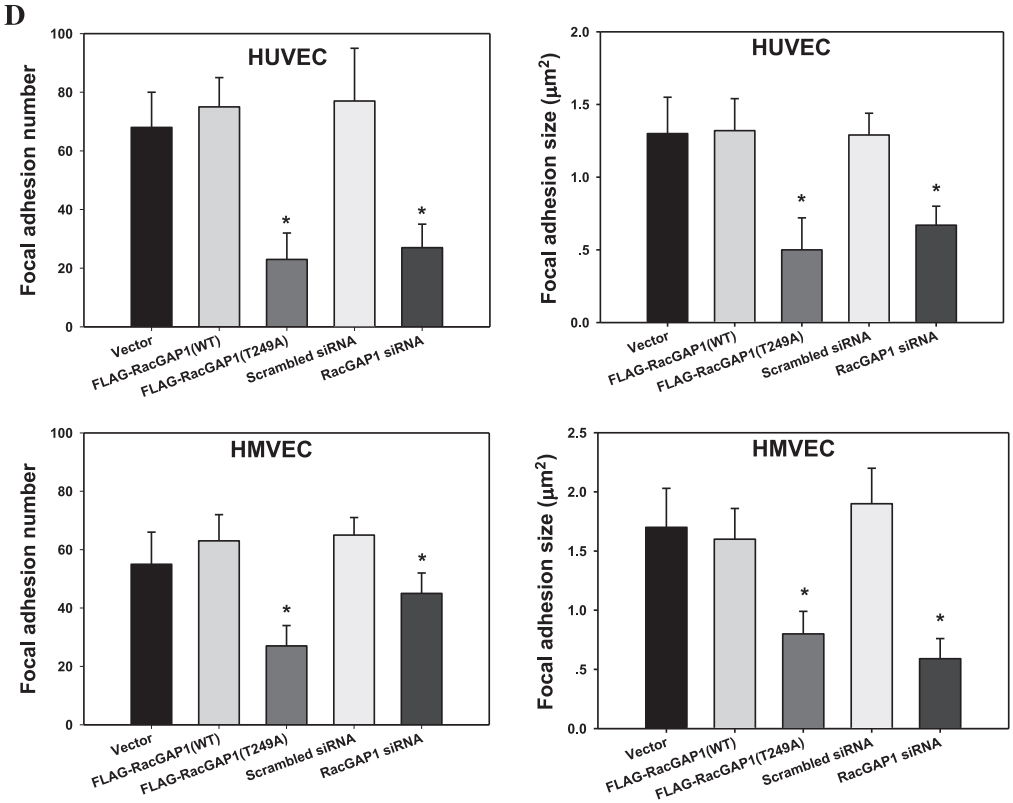

Fig. 3. (*continued*).

3.3. The regulation of RhoA activation and focal adhesion assembly through RacGAP1 signaling facilitated Lu1205 transendothelial migration

Since RacGAP1 is mostly recruited to cell adhesion sites and related to Rho GTPase activity modulation [24,25], the activities of endothelial RhoA, FAK and paxillin in responses to melanoma adhesion were investigated. Lu1205 adhesion induced GTP association of RhoA, Y397 phosphorylation of FAK and Y118 phosphorylation of paxillin in a time-dependent manner (Fig. 3A). Lu1205-induced RhoA, FAK and paxillin activations were diminished in HUVECs or HMVECs receiving FLAG-RacGAP1^{T249A} or RacGAP1 siRNA (Fig. 3B). Since cytoskeletal structure may play important roles in endothelial barrier function, we analyzed the dynamics of actin cytoskeleton and focal adhesion. In vector or scrambled siRNA-transfected control HUVECs, Lu1205 cells induced stress fiber formation with thick actin bundles traversing the cell bodies (Fig. 3C). In FLAG-RacGAP1^{WT}-transfected HUVECs, the stress fibers became more robust and organized. In contrast, FLAG-RacGAP1^{T249A} or RacGAP1 siRNA-transfected HUVECs exhibited filamentous actin assembled around cell periphery, with only a few thin stress fibers located within cell body. Paxillin is a multidomain scaffold adaptor protein which recruits structural and signaling molecules to focal adhesions. It plays an important role in transducing signals for cell adhesion and cytoskeletal reorganization [26]. By staining paxillin, it was shown that control and FLAG-RacGAP1^{WT}-transfected cells displayed bright punctate focal adhesions which were colocalized with the end of thick stress fibers. However, in FLAG-RacGAP1^{T249A} or RacGAP1 siRNA-transfected HUVECs, small focal adhesions were visible at cell periphery and almost disengaged with thin stress fibers. Overexpressing FLAG-RacGAP1^{T249A} or silencing with RacGAP1 siRNA decreased the size and number of focal adhesions in HUVECs or HMVECs (Fig. 3D). These data implied that RhoA activation, focal adhesion assembly and cytoskeletal rearrangement were located in the downstream of RacGAP1 activation.

3.4. Overexpression of FAK-related non-kinase affected Lu1205 transendothelial migration

Focal adhesion molecules may be associated with actin fibers to regulate cell contractility and localize to adherens junctions to regulate endothelial barrier functions [17,27–29]. To determine whether focal adhesion molecule activation downstream of RacGAP1 signaling may regulate melanoma transmigration, a FAK-related non-kinase (FRNK), which is a dominantly negative regulator of FAK function by competing with native FAK for focal adhesion binding sites, was cloned and transfected into HUVECs and HMVECs. FRNK overexpression considerably prevented MDA-MB-231 transmigration (Fig. 4A). Furthermore, Lu1205 cell-induced changes of endothelial permeability with respect to time were not observed in cells overexpressing FRNK (Fig. 4B). FRNK abrogated time-dependent junction breakdown mediated by Lu1205 adhesion (Fig. 4C). These data suggests that a focal adhesion-mediated signaling downstream of RacGAP1 contributed to Lu1205-induced endothelial junction breakdown and melanoma extravasation.

4. Discussion

Hematogenous dissemination of melanoma requires tumor adherence to endothelium and dissociation of adherens junctions [4,5,30,31]. Previous studies indicated that signaling transduced from tumor cells may promote the dissociation of β-catenin and VE-cadherin complexes and disrupt endothelial barrier function [1,32] . However, the molecular mechanism of this process remains

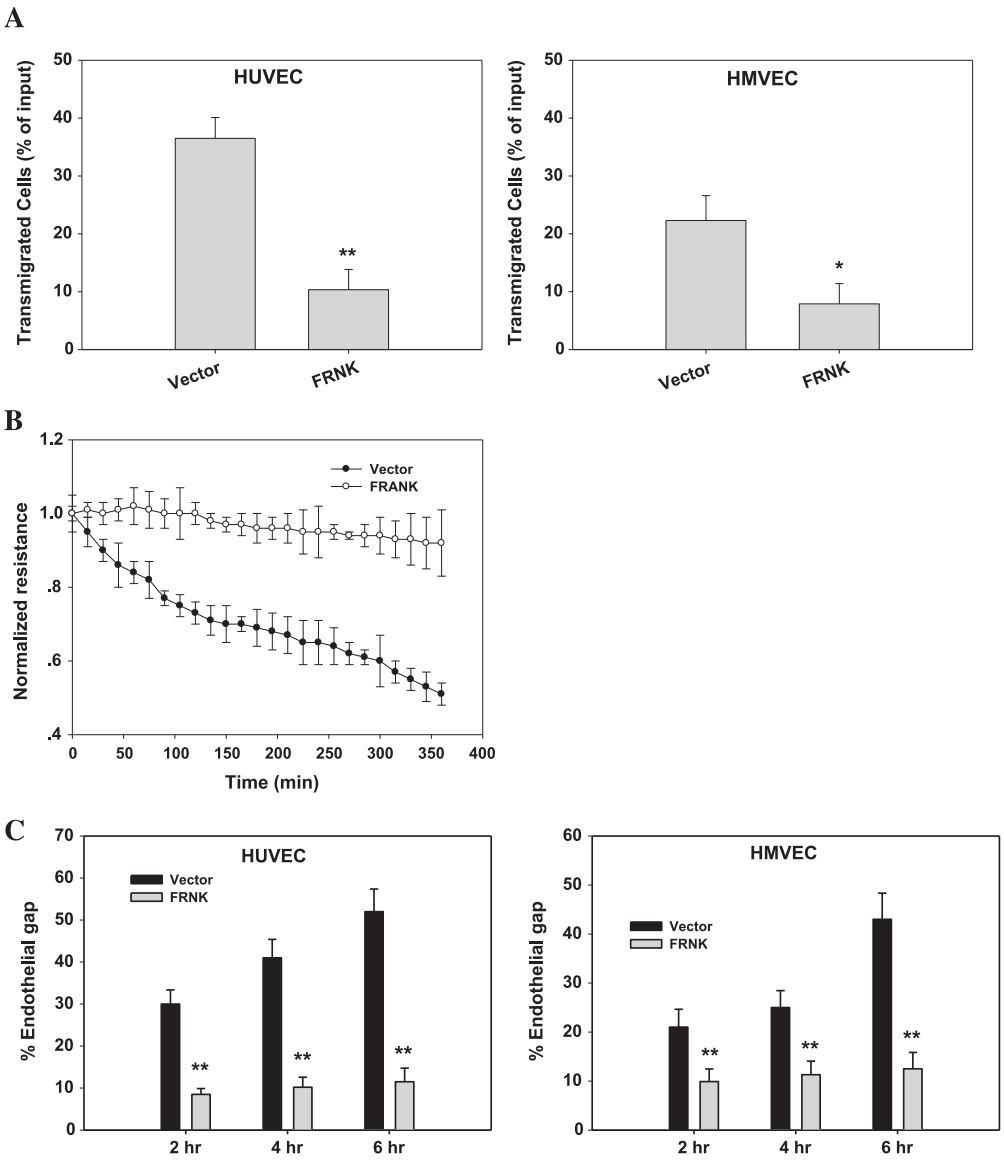

Fig. 4. Focal adhesion formation was required for melanoma migration and endothelial permeability alternation. HUVECs or HMVECs were transfected with vector or FRANK before being co-cultured with Lu1205 cells for 6 h. Then, Lu1205 transendothelial migration (A) and the temporal changes of TER (B) were measured. Percentage of endothelial gap formation induced by co-culturing HUVEC or HMVEC transfectants with Lu1205 for different time periods were quantified (C). Data were expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

elusive. In the current study, we found that endothelial RacGAP1, as a key regulator of endothelial permeability, mediated the activation of RhoA and regulated focal adhesion assembly during melanoma transendothelial migration. RacGAP1 is implicated in cytoskeletal organization in the mitotic spindle formation during cytokinesis [21]. RacGAP1 may function as nuclear chaperon mediating STAT nuclear transportation [33]. However, few studies linked its function to cancer extravasation and metastasis. Recently, it was reported that phosphorylation of RacGAP1 promoted its recruitment to pseudopodia and reorganization of actin cytoskeleton in a RhoA activation-dependent manner [24]. In agreement with this study, we found that RacGAP1 knockdown reduced RhoA activation. Therefore, in this context, we predict that RacGAP1 activation in response to tumor adhesion may limit Rac activity and elevate RhoA activity. RhoA has been shown to enhance cell contractility, while Rac could suppress this process and stabilize cell–cell junction. Given the fundamental role that RhoA plays in regulating actin polymerization and cellular behaviors, it is perhaps that RacGAP1 coordinated RhoA activities spatially to regulate actomyosin-mediated cell contractility and junction breakdown [19,34].

In the current study, we revealed that focal adhesion molecules, like FAK and paxillin, were downstream of RacGAP1 activation. It is unknown whether RacGAP1 mediated FAK activation and focal adhesion formation directly or indirectly. Accumulating evidence indicates that IQGAP1, an IQ-domain containing protein, play important roles in mediating actin cytoskeletal rearrangement, cell retraction and endothelial junction disruption [35–38]. IQGAP1 serves a scaffold protein recruiting RhoGTPase to cell–cell junctions [39]. Recent studies suggest that IQGAP1 can be associated with T249 phosphorylated RacGAP1 at cell adhesion sites [23]. It is possible that melanoma adhesion may trigger translocation of RacGAP1 to adherens junctions where it can recruit IQGAP1, RhoGTPases and focal adhesion proteins. This conjecture may be supported by the current study, since we found that T249 mutation on RacGAP1 abrogated RhoA activation and reduced endothelial permeability. Recent studies showed that FAK may directly

phosphorylate VE-cadherin at tyrosine 658 [16]. Loss of FAK enhanced endothelial barrier functions. Therefore, FAK recruited and activated by RacGAP1 may phosphorylate VE-cadherin and disrupt VE-cadherin and β-catenin binding. On the other hand, FAK may mediate stress fiber formation and cell retraction. All of these facts highlight the importance of RacGAP1 during junction breakdown and tumor transmigration. The next challenge will be to investigate the upstream signaling responsible for RacGAP1 phosphorylation.

In conclusion, we reported a signaling pathway linking RacGAP1 activation to focal adhesion formation and endothelial junction breakdown that controls melanoma transendothelial migration. We envision RacGAP1 as a key node of a complex regulatory network, integrating melanoma contact-initiated signals to regulate endothelial barrier function.

Acknowledgments

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References

[1] P.L. Tremblay, F.A. Auger, J. Huot, Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases, Oncogene 25 (2006) 6563–6573.
[2] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[3] K. Konstantopoulos, S.N. Thomas, Hematogenous metastasis: roles of CD44v and alternative sialofucosylated selectin ligands, Adv. Exp. Med. Biol. 705 (2011) 601–619.
[4] D. Wirtz, K. Konstantopoulos, P.C. Searson, The physics of cancer: the role of physical interactions and mechanical forces in metastasis, Nat. Rev. Cancer 11 (2011) 512–522.
[5] K. Konstantopoulos, S.N. Thomas, Cancer cells in transit: the vascular interactions of tumor cells, Annu. Rev. Biomed. Eng. 11 (2009) 177–202.
[6] T.C. Tsou, S.C. Yeh, F.Y. Tsai, H.J. Lin, T.J. Cheng, H.R. Chao, L.A. Tai, Zinc oxide particles induce inflammatory responses in vascular endothelial cells via NF-kappaB signaling, J. Hazard. Mater. 183 (2010) 182–188.
[7] L. Su, L. Han, F. Ge, S.L. Zhang, Y. Zhang, B.X. Zhao, J. Zhao, J.Y. Miao, The effect of novel magnetic nanoparticles on vascular endothelial cell function in vitro and in vivo, J. Hazard. Mater. 235–236 (2012) 316–325.
[8] M.K. Hoelzle, T. Svitkina, The cytoskeletal mechanisms of cell–cell junction formation in endothelial cells, Mol. Biol. Cell 23 (2012) 310–323.
[9] S. Huveneers, J. Oldenburg, E. Spanjaard, G. van der Krogt, I. Grigoriev, A. Akhmanova, H. Rehmann, J. de Rooij, Vinculin associates with endothelial VE-cadherin junctions to control force-dependent remodeling, J. Cell Biol. 196 (2012) 641–652.
[10] E. Dejana, F. Orsenigo, M.G. Lampugnani, The role of adherens junctions and VE-cadherin in the control of vascular permeability, J. Cell Sci. 121 (2008) 2115–2122.
[11] E. Dejana, E. Tournier-Lasserve, B.M. Weinstein, The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications, Dev. Cell 16 (2009) 209–221.
[12] M.D. Potter, S. Barbero, D.A. Cheresh, Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state, J. Biol. Chem. 280 (2005) 31906–31912.
[13] A.P. Adam, A.L. Sharenko, K. Pumiglia, P.A. Vincent, Src-induced tyrosine phosphorylation of VE-cadherin is not sufficient to decrease barrier function of endothelial monolayers, J. Biol. Chem. 285 (2010) 7045–7055.
[14] M. Giannotta, M. Trani, E. Dejana, VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity, Dev. Cell 26 (2013) 441–454.
[15] T. Borbiev, A. Birukova, F. Liu, S. Nurmukhambetova, W.T. Gerthoffer, J.G. Garcia, A.D. Verin, P38 MAP kinase-dependent regulation of endothelial cell permeability, Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L911–L918.
[16] C. Jean, X.L. Chen, J.O. Nam, I. Tancioni, S. Uryu, C. Lawson, K.K. Ward, C.T. Walsh, N.L. Miller, M. Ghassemian, P. Turowski, E. Dejana, S. Weis, D.A. Cheresh, D.D. Schlaepfer, Inhibition of endothelial FAK activity prevents tumor metastasis by enhancing barrier function, J. Cell Biol. 204 (2014) 247–263.
[17] M.H. Wu, Endothelial focal adhesions and barrier function, J. Physiol. 569 (2005) 359–366.
[18] F.E. Nwariaku, P. Rothenbach, Z. Liu, X. Zhu, R.H. Turnage, L.S. Terada, Rho inhibition decreases TNF-induced endothelial MAPK activation and monolayer permeability, J. Appl. Physiol. 95 (2003) (1985) 1889–1895.
[19] B. Wojciak-Stothard, A.J. Ridley, Rho GTPases and the regulation of endothelial permeability, Vascul. Pharmacol. 39 (2002) 187–199.
[20] B. Wojciak-Stothard, S. Potempa, T. Eichholtz, A.J. Ridley, Rho and Rac but not Cdc42 regulate endothelial cell permeability, J. Cell Sci. 114 (2001) 1343–1355.
[21] F. Oceguera-Yanez, K. Kimura, S. Yasuda, C. Higashida, T. Kitamura, Y. Hiraoka, T. Haraguchi, S. Narumiya, Ect2 and MgcRacGAP regulate the activation and function of Cdc42 in mitosis, J. Cell Biol. 168 (2005) 221–232.
[22] T. Kitamura, T. Kawashima, Y. Minoshima, Y. Tonozuka, K. Hirose, T. Nosaka, Role of MgcRacGAP/Cyk4 as a regulator of the small GTPase Rho family in cytokinesis and cell differentiation, Cell Struct. Funct. 26 (2001) 645–651.
[23] G. Jacquemet, M.R. Morgan, A. Byron, J.D. Humphries, C.K. Choi, C.S. Chen, P.T. Caswell, M.J. Humphries, Rac1 is deactivated at integrin activation sites through an IQGAP1-filamin-A-RacGAP1 pathway, J. Cell Sci. 126 (2013) 4121–4135.
[24] G. Jacquemet, D.M. Green, R.E. Bridgewater, A. von Kriegsheim, M.J. Humphries, J.C. Norman, P.T. Caswell, RCP-driven alpha5beta1 recycling suppresses Rac and promotes RhoA activity via the RacGAP1–IQGAP1 complex, J. Cell Biol. 202 (2013) 917–935.
[25] A. Ratheesh, G.A. Gomez, R. Priya, S. Verma, E.M. Kovacs, K. Jiang, N.H. Brown, A. Akhmanova, S.J. Stehbens, A.S. Yap, Centralspindlin and alpha-catenin regulate Rho signalling at the epithelial zonula adherens, Nat. Cell Biol. 14 (2012) 818–828.
[26] M.C. Brown, C.E. Turner, Paxillin: adapting to change, Physiol. Rev. 84 (2004) 1315–1339.
[27] K.M. Arnold, Z.M. Goeckeler, R.B. Wysolmerski, Loss of focal adhesion kinase enhances endothelial barrier function and increases focal adhesions, Microcirculation 20 (2013) 637–649.
[28] X. Li, J. Regezi, F.P. Ross, S. Blystone, D. Ilic, S.P. Leong, D.M. Ramos, Integrin alphavbeta3 mediates K1735 murine melanoma cell motility in vivo and in vitro, J. Cell Sci. 114 (2001) 2665–2672.
[29] S.K. Mitra, D.A. Hanson, D.D. Schlaepfer, Focal adhesion kinase: in command and control of cell motility, Nat. Rev. Mol. Cell Biol. 6 (2005) 56–68.
[30] P. Zhang, C. Goodrich, C. Fu, C. Dong, Melanoma upregulates ICAM-1 expression on endothelial cells through engagement of tumor CD44 with endothelial E-selectin and activation of a PKCalpha-p38-SP-1 pathway, FASEB J. (2014).
[31] P. Khanna, C.Y. Chung, R.I. Neves, G.P. Robertson, C. Dong, CD82/KAI expression prevents IL-8-mediated endothelial gap formation in late-stage melanomas, Oncogene (2013).
[32] S. Weis, J. Cui, L. Barnes, D. Cheresh, Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis, J. Cell Biol. 167 (2004) 223–229.
[33] T. Kawashima, Y.C. Bao, Y. Nomura, Y. Moon, Y. Tonozuka, Y. Minoshima, T. Hatori, A. Tsuchiya, M. Kiyono, T. Nosaka, H. Nakajima, D.A. Williams, T. Kitamura, Rac1 and a GTPase-activating protein, MgcRacGAP, are required for nuclear translocation of STAT transcription factors, J. Cell Biol. 175 (2006) 937–946.
[34] J. McCormack, N.J. Welsh, V.M. Braga, Cycling around cell–cell adhesion with Rho GTPase regulators, J. Cell Sci. 126 (2013) 379–391.
[35] S. Foroutannejad, N. Rohner, M. Reimer, G. Kwon, J.M. Schober, A novel role for IQGAP1 protein in cell motility through cell retraction, Biochem. Biophys. Res. Commun. 448 (2014) 39–44.
[36] S. Krishnan, G.E. Fernandez, D.B. Sacks, N.V. Prasadarao, IQGAP1 mediates the disruption of adherens junctions to promote *Escherichia coli* K1 invasion of brain endothelial cells, Cell. Microbiol. 14 (2012) 1415–1433.
[37] M. Yamaoka-Tojo, T. Tojo, H.W. Kim, L. Hilenski, N.A. Patrushev, L. Zhang, T. Fukai, M. Ushio-Fukai, IQGAP1 mediates VE-cadherin-based cell–cell contacts and VEGF signaling at adherence junctions linked to angiogenesis, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 1991–1997.
[38] J.M. Mataraza, M.W. Briggs, Z. Li, A. Entwistle, A.J. Ridley, D.B. Sacks, IQGAP1 promotes cell motility and invasion, J. Biol. Chem. 278 (2003) 41237–41245.
[39] M.D. Brown, D.B. Sacks, IQGAP1 in cellular signaling: bridging the GAP, Trends Cell Biol. 16 (2006) 242–249.