BRAF increases endothelial cell stiffness through reorganization of the actin cytoskeleton

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Abstract
The dynamics of the actin cytoskeleton and its connection to endothelial cell–cell junctions determine the barrier function of endothelial cells. The proper regulation of barrier opening/closing is necessary for the normal function of vessels, and its dysregulation can result in chronic and acute inflammation leading to edema formation. By using atomic force microscopy, we show here that thrombin-induced permeability of human umbilical vein endothelial cells, associated with actin stress fiber formation, stiffens the cell center. The depletion of the MEK/ERK kinase BRAF reduces thrombin-induced permeability prevents stress fiber formation and cell stiffening. The peripheral actin ring becomes stabilized by phosphorylated myosin light chain, while cofilin is excluded from the cell periphery. All these changes can be reverted by the inhibition of ROCK, but not of the MEK/ERK module. We propose that the balance between the binding of cofilin and myosin to F-actin in the cell periphery, which is regulated by the activity of ROCK, determines the local dynamics of actin reorganization, ultimately driving or preventing stress fiber formation.

KEYWORDS
actin cytoskeleton, atomic force microscopy, BRAF RNAi, endothelial monolayer, intercellular gaps, permeability

Abbreviations: AFM, atomic force microscopy; bFGF, basic Fibroblast Growth Factor; CAB, circumferential actin bundles; FBS, fetal bovine serum; FI, fluorescence intensity; HUVEC, human umbilical vein endothelial cell; IF, immunofluorescence; MLC, myosin light chain; MLCK, myosin light chain kinase; p-cofilin, phosphorylated cofilin; pERK, phosphorylated ERK; pMLC, phosphorylated form of MLC; ROCK, Rho-dependent kinase; RSF, radial stress fibers; RT, room temperature; TBS-T, 1% Tween-20 in TBS; WB, western blotting.

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1 INTRODUCTION

The barrier function of the endothelium is tightly regulated, and its failure can lead to chronic inflammation as well as an increased metastatic risk.1 Weakening of endothelial cell–cell (junctional) adhesion can be triggered by cytokines, growth factors, protease-activated receptor (also known as thrombin receptor) agonists, and several other molecules reviewed in Debreczeni et al.2 Under resting condition, VE-cadherin-factors, protease-activated receptor (also known as thrombin receptor) agonists, and several other molecules reviewed in Debreczeni et al.2 Under resting condition, VE-cadherin-based linear adherens junctions are stabilized by circumferential actin bundles (CAB). Permeability-increasing agents induce the reorganization of the actin cytoskeleton and the formation of radial stress fibers (RSF), which is accompanied by gap formation between two endothelial cells.3 CAB formation is stabilized by the local activity of Rac/Rap at the membrane, while Rho activity is necessary for the contraction of actin bundles in the cell center.

Downstream of Rho, the Rho-dependent kinase ROCK exerts its effect on the actin cytoskeleton in two ways.4 First, through the activation of LIMK, ROCK increases the phosphorylation of coflin, thereby preventing actin depolymerization mediated by unphosphorylated coflin. Second, ROCK can enhance actin filament contraction by phosphorylating myosin light chain (MLC) directly or by inactivating the MLC phosphatase, MYPT1. Thrombin also activates myosin light chain kinase (MLCK), which can directly phosphorylate MLC just as ROCK can. In a few cell types, ROCK and MLCK were proposed to act at distinct locations within the cell. It was reported that ROCK phosphorylates MLC at the central stress fibers, while MLCK is responsible for the peripheral phosphorylation of MLC.6,7

Thrombin stimulation initially increases RhoA activity in the cell periphery, followed by a more sustained RhoA activation along the stress fibers.8 This observation is in line with the spatio-temporal phosphorylation and activation of MLC.9 Mechanistically, it has been proposed that at early time points, increased MLC phosphorylation in the cell periphery is required for ROCK-dependent actin reorganization from CAB to RSF. Interestingly, Rho activity is also needed to seal gaps formed between two endothelial cells, and indeed, the spatio-temporal activity of RhoA can be correlated with gap closure in the cell periphery.8 Local, peripheral RhoA signaling through one of the ROCK isoforms, ROCKII is important for preventing vascular leakage during leukocyte diapedesis.10 At the same time, rapid Rac1 inactivation downstream of thrombin happens at cell–cell junctions,11 in line with the opposing activities of RhoA and Rac at the junctions.12 Thus, Rho activity is subject to tight spatio-temporal regulation at endothelial cell–cell junctions resulting in the fine control of their opening and closing.

The activity of ROCK was shown to be regulated by the MEK/ERK pathway in different cell types. The main MEK/ERK activator BRAF contributes to stress fiber formation in fibroblasts13 and endothelial cells.14,15 Mechanistically, while in fibroblast MEK re-activation can restore stress fiber formation, in mouse endothelial cells the effect of BRAF on the actin cytoskeleton is linked to the heterodimer formation between BRAF and RAF1.15 RAF1 is essential for the delivery of ROCKII to cell–cell junctions,16 while BRAF reduces the binding of RAF1 to ROCKII at cell–cell junctions. In the absence of BRAF, more ROCKII is delivered to cell–cell junctions by RAF115 and this results in a less permeable endothelial monolayer and decreased melanoma metastasis formation. Importantly, the permeability phenotype cannot be phenocopied by MEK inhibition. In human umbilical vein endothelial cells (HUVECs), MEK inhibition also does not change thrombin-induced permeability.17 Therefore, the question arises whether in human endothelial cells BRAF can fulfill a similar function as in mouse endothelial cells through the regulation of ROCK localization and activity.

Reorganization of the actin cytoskeleton modulates cellular stiffness.18,19 The effect of permeability-increasing agents on the actin cytoskeleton can be correlated with an increased overall stiffness, and this is realized through stiffening of the cell center.20 By contrast, barrier-protecting agents decrease overall cell stiffness but slightly increase it in the cell periphery. The adhesion of leukocytes also causes local cellular stiffening.19 At the molecular level, the actin crosslinker α-actinin 4 identified downstream of the adhesion receptor, ICAM-1 is necessary for an efficient leukocyte transmigration and its depletion was shown to decrease peripheral stiffness.21 The question arises why permeability increase is accompanied by stiffening of the cell center, while during leukocyte transmigration it is the cell periphery that stiffens. One possible explanation for this apparent discrepancy is that at the site of transmigration plasma leakage is limited rather than increased22 and this might be achieved through local (peripheral) stiffening. Since RhoA and ROCKII activity is necessary to limit plasma leakage in the cell periphery and RhoA also plays a role during the closure of thrombin-induced gaps, the question arises whether local RhoA and ROCK activity correlates with peripheral stiffening. Here, we aimed to determine how BRAF regulates human endothelial cell stiffness upon permeability induction, and how BRAF-dependent local actin remodeling correlates with changes in stiffness.
2 | MATERIALS AND METHODS

2.1 | Reagents

2.1.1 | Primary antibodies for western blotting (WB)

BRAF, Cat# 14814S; coflin (both WB and IF), Cat# 5175S; ERK, Cat# 9102; pERK, Cat# 9101; GAPDH, Cat# 97166S; MLC, Cat# 8505S; p-cofilin (both WB and IF), Cat# 3313S; pMLC (Thr18/Ser19), Cat# 3674S; all from Cell Signaling. ROCK, Cat# 04–841 from Merck Life Science.

2.1.2 | Primary antibodies for immunofluorescence (IF)

VE-cadherin, Cat# 2500S; PECAM-1, Cat# 3528S; pMLC (Ser19), Cat# 3675S; all from Cell Signaling.

2.1.3 | Secondary antibodies for WB

Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L), Cat#115–035-003; Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L), Cat# 111–035-003; all from Jackson ImmunoResearch.

2.1.4 | Secondary antibodies for IF

Chicken anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Cat# A21441; Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546, Cat# A11003; both from ThermoFisher Scientific; Phalloidin CruzFluor™ 647 Conjugate, Cat# sc-363 797 from Santa Cruz Biotechnology.

2.1.5 | siRNAs

siControl: ON-TARGETplus Non-targeting Control Pool, Cat# D001810-10-20; siBRAF: ON-TARGETplus Smart Pool Human BRAF, Cat# L-003460-00-0020; both from Dharmacon.

2.1.6 | Bacterial strains

TOP10 chemically competent E. Coli, Cat# C404010; Stbl3 chemically competent E. Coli, Cat# C737303 were from ThermoFisher Scientific.

2.1.7 | Chemicals for cell culture

Basic Fibroblast Growth Factor, Cat# F0291; Gelatin, type B, 2% solution, Cat# G1393; Heparin, Cat# H3149; Hydrocortison, Cat# H0396; Polydopamine, Cat# H8502; Vitamine C, Cat# A4544; thrombin, Cat# 605195-100U, all from Sigma. AIMV medium, #Cat 12055091; Chemically Defined Lipid Concentrate, Cat# 11905031; DMEM, Cat# 10313021; EGF, Cat# PHG0311; GlutaMAX Supplement, Cat# 35050038; HBSS, Cat# 14025050; HEPES, 1 M Buffer Solution, Cat# 15630049; Insulin-Transferrin-Selenium, Cat# 41400045; L-Glutamine, Cat# 25030081; MCDB-131 medium, Cat# 10372019; Penicillin–Streptomycin, Cat# 15140148; all from ThermoFisher Scientific. Fetal Bovine Serum, Cat# P40-39500 from PAN Biotech.

2.1.8 | Inhibitors

MEK inhibitor, U0126, Cat# 662005 from Merck Life Science (Sigma); ROCK inhibitor, Y27632, Cat# 13624S from Cell Signaling.

2.1.9 | Cultured cells/cell lines

HUVECs were purchased from Caltag Medsystems (UK) (Cat# ZHC-2301) and were cultured in MCDB medium, supplemented with 5% fetal bovine serum (FBS), 1% penicillin–streptomycin, 1% Chemically Defined Lipid Concentrate, 1% HEPES, 1% Gluta-MAX Supplement, 0.3% insulin–transferrin–selenium, 1 ng/ml basic Fibroblast Growth Factor (bFGF), 2 ng/ml EGF, 5 μg/ml vitamin C, 250 mM hydrocortisone and 7.5 U/ml heparin. Tissue culture dishes were coated with 0.5% gelatin for proper attachment of HUVECs. AIMV medium was supplemented with 1% FBS, 1 ng/ml bFGF, 2 ng/ml EGF and 7.5 U/ml heparin. HEK293T cells used for lentiviral production were cultured and transfected with the appropriate plasmids as described in Hollósi et al.23

2.2 | Methods

2.2.1 | Lenti-viral packaging

Lenti-viral packaging of shControl/shBRAF constructs was performed using HEK293T cells. Transfection was carried out using PEI as a transfection reagent, which was prepared according to Aricescu et al.24 2.8×10^6 HEK cells were seeded in T25 flask the day before transfection. The plasmid mixture contained 4 μg SGEp-shControl/shBRAF plasmid, 1.5 μg pCMV-VSV-G, 0.75 μg pRSV-Rev
and 0.75μg pMDLg/pRRE virus plasmids. PEI was used in 1:2 ratio. HEK transfection medium was exchanged to cultured DMEM medium 3–4 h after transfection. After 24h HUVEC medium was applied on the transfected HEK cells. Virus-containing supernatant was harvested after a total of 48h and filtered through 0.45μm filter.

For transduction of HUVECs, cells were cultured in 50% HUVEC medium and 50% viral supernatant for 24h, supplemented with polybrene (Santa Cruz Biotechnology, Cat# sc-134220, 4μg/ml final concentration). For transduction of HUVECs, the virus was mixed with trypsinized HUVECs and 1.5–2×10^5 cells were seeded on a cell culture plate (growth area 9 cm^2).

2.2.2 | siRNA transfection

siRNA transfection was carried out in OPTI-MEM (ThermoFisher Scientific, Cat# 31985062) by using 6μl Lipofectamine RNAiMAX/ml OPTI-MEM (ThermoFisher Scientific, Cat# 13778030) and 25 nM siRNA. The mixtures were prepared according to the manufacturer's instructions. Cells were incubated with the siRNA-RNAiMAX mixture for 4 h, trypsinized and seeded according to the type of experiment. 8–10×10^4 were seeded in 24-well plates for western blot analysis, and 6×10^4 cells were seeded on 8-well chamber slide (iBidi, Cat# 80827) for immunofluorescence staining.

2.2.3 | Thrombin and inhibitor treatments

HUVECs were cultured in complete MCDB medium for 48h after siRNA treatment. The day before thrombin treatment, they were incubated for 16h with a 50–50% mixture of complete MCDB and complete AIMV, then for another hour in complete AIMV medium (if any inhibitor was applied, it was added in the AIMV medium) and then stimulated with 1 U/ml thrombin for different periods of time (except for the permeability and transmigration assays, where a thrombin concentration of 3 U/ml was used). Both U0126 and Y27632 were applied at a concentration of 10 μM for an hour in complete AIMV before thrombin treatment.

2.2.4 | Immunofluorescence staining of fixed monolayers

Endothelial cells grown on 8-well chamber slide were fixed with Image-iT™ Fixative Solution (ThermoFisher Scientific, Cat# R37814) for 15min. Subsequently, cells were washed with HBSS, permeabilized (0.25% Triton X-100 in TBS-T, 10 min RT), blocked (1% BSA in TBS-T, 1h RT), and incubated with the primary antibodies (dilutions prepared in 1% BSA-TBS-T for VE-cadherin – 1:400, pMLC – 1:200; PECAM-1-1:2000, p-cofilin-1:100, cofilin-1:200, incubation was done overnight at 4 °C). After thorough washing in TBS-T (1% Tween-20 in TBS), cells were stained simultaneously with the appropriate secondary antibodies (dilutions were prepared as 1:2000) and phalloidin (1:1000 in 1% BSA-TBS-T) for 1h at RT, washed in TBS-T and PBS, then stained with Hoechst (Cat# 62249, Thermo Scientific) for 5 min, and finally washed in PBS prior to imaging. Confocal imaging was performed on a Nikon Ti2 inverted microscope (Expertline, Abberior Instruments, Göttingen Germany). The field of view for imaging was a 120μm x 120μm area and pictures were taken by using a 60x lens (numerical aperture: 1.40, oil).

2.2.5 | Immunoblotting

Cells were harvested in 25mM HEPES, pH 7.4, 150mM NaCl, 1mM EGTA, 1% NP-40, 10% glycerol, supplemented with the following protease and phosphatase inhibitors: 10mM sodium pyrophosphate, 10mM sodium fluoride, 5mM sodium vanadate, 1mM PMSF and complete, EDTA-free protease inhibitor cocktail (Sigma). Lysates were centrifuged with 5000xg for 5 min at 4°C, and the supernatant was snap frozen for further immunoblotting.

Proteins were separated using standard SDS-PAGE gel electrophoresis with 12% SDS-PAGE gels, transferred to PVDF membranes for immunoblot analysis using a wet blot transfer system (BioRad), and stained with specific primary antibodies as indicated in each figure. After HRP-conjugated secondary antibody incubation the membranes were incubated with chemiluminescence substrate and developed on Hyperfilms. Bands were quantified by using ImageJ 1.53c.

2.2.6 | Transendothelial migration assay

6×10^4 HUVECs were cultured on gelatin-coated 96-well inserts (8-μm pore size, Cat# 89089–938, VWR) for 48h, starved overnight with 50–50% MCDB-AIMV medium, then the medium was replaced with AIMV for 1 h before applying melanoma cells on top of them. The medium in the lower chamber was the growth medium of HUVECs. A375 melanoma cells (1×10^5) stained with Oregon Green dye (Cat# C34555, ThermoFisher Scientific) were added to the upper chamber and incubated for 4 h with or without thrombin (3 U/ml). Transmigrated cells were dissociated from the lower part of the chamber by using a cell dissociation buffer (5 mM EDTA in PBS) and fluorescence intensities were measured on a CLARIOstar microplate
reader (BMG LABTECH, excitation: 483 nm, emission: 530 nm). The integrity of the monolayers was determined by orange CellMask staining (Cat# C10045). Experiments were performed in triplicates.

### 2.2.7 Permeability measurements

Permeability tests were carried out using a modified version of the recently developed XPerT technique. Confluent layers of HUVECs were seeded onto 96-well plates pre-coated with 250 μg/ml biotinylated gelatin and were cultured in AIMV medium for 2 days. After thrombin treatment (3 U/ml), Streptavidin-Alexa488 (2 μg/ml, Life Technologies) was added to each well for 2 min. Cells were fixed with 1% paraformaldehyde-PBS and two images of each well were taken by using an Olympus IX-81 fluorescence microscope and an Olympus XM-10 camera. The size of the stained area was determined on each image by quantitative image analysis using the CellP software (Olympus).

### 2.2.8 Atomic force microscopy

Fluorescently labeled cells and molecules were visualized with an Olympus IX81 motorized epifluorescence microscope (10x, 0.3 numerical aperture objective lens) and AFM combination. The sample was positioned on a special, double XY microscope stage, which is part of the AFM system (MFP-3D, Asylum Research, Santa Barbara, CA). The lower, mechanical stage permits the movement of the AFM head (together with the upper, sample stage) relative to the optical axis. The upper, sample stage is a closed-loop XY-piezo stage. To obtain spatially synchronized AFM and fluorescence images, the cantilever tip was aligned with the optical axis by manually positioning the AFM head with the lower stage under video control.

AFM imaging was performed in contact mode with an MFP3D AFM using Bruker, MSCT-A probes (nominal typical spring constant = 70 pN/nm). Cantilevers were calibrated by the thermal method. Cell monolayer samples were grown on circular microscope slides, which were fixed after thrombin treatment with 4% PFA and washed two times with PBS, then they were mounted in the BioHeater module of the AFM. Imaging and force spectroscopy were carried out in a temperature-controlled liquid environment at 37°C. Live cell AFM imaging was also carried out without thrombin treatment in AIMV medium, at 37°C. Since stiffness is temperature dependent, and we wanted to compare the stiffness of fixed and live cells, we carried out both (fixed and live cell measurements) at 37°C. The epifluorescence image of the EGFP-expressing cells was used to find optimal regions for AFM imaging. First an AFM image was taken, then in situ force spectroscopy was carried out collecting 100 force curves on selected 3x3 μm regions of central and peripheral parts of endothelial cell surfaces (force mapping). The individual force curves were recorded with a vertical Z-piezo movement speed of 1 μm/s, until the force set point (5 nN for fixed cells and 1 nN for live cells) was reached, then the tip was retracted. Elastic moduli were obtained by fitting the indentation curves with the blunted pyramidal model as described in Rico et al. For the calculations, the irregular pyramid shape with a semi-included angle of 20°, and with a spherical cap radius of 10 nm was used as described for the Bruker, MSCT-A probe. Poisson ratio of the tip and the sample was set to 0.2 and 0.5, respectively.

### 2.2.9 Quantification of fluorescence intensities

Fluorescence intensity (FI) of cofilin staining was quantified using ImageJ 1.53c. The periphery of the cell was defined by using the selection scale factor 0.8 (both for x and y direction) built in ImageJ. This scale factor divided the area of the cell to give a periphery to center ratio of 1:2. FI of cofilin was measured within the whole cell (by using the composite image with the PECAM membrane staining) and in the center of the cell (by using the scale factor 0.8). Then the FI measured in the center was subtracted from the FI of the “whole cell” to get the FI in the periphery. The measured (center) or calculated (periphery) FIs were normalized to the corresponding area, and the ratio of periphery to center FIs (normalized to the area) was calculated for each cell. At least 15 cells were used to calculate the ratio for each condition.

Data are presented in all figures as mean ± SEM for biological replicates. Statistical analyses were carried out in GraphPad Prism 4 (version 4.01). Significance was determined by a two-tailed t-test in Microsoft Excel. Differences between groups were considered statistically significant if \( p < .05 \).

### 3 RESULTS

#### 3.1 Thrombin-induced permeability and melanoma transmigration are reduced upon BRAF knockdown and are accompanied by diminished MLC and increased cofilin phosphorylation

We have previously shown that ablation of BRAF in mouse endothelial cells improves the barrier function of
the monolayer and decreases melanoma metastasis formation. In this study, we used BRAF RNAi to test its effect on the permeability of HUVEC monolayers exposed to thrombin. The permeability of BRAF RNAi-treated monolayers was slightly increased compared to the control RNAi-treated monolayer (Figure 1A). Thrombin stimulation increased permeability to a lesser extent in BRAF-deficient HUVEC monolayers than in BRAF-proficient cells (Figure 1B). Consistent with this, thrombin treatment increased the paracellular migration of the human melanoma cell line A375 across BRAF-proficient but not BRAF-deficient HUVEC monolayers (Figure 1C). These results indicate that BRAF supports endothelial permeability and paracellular migration of melanoma cells in a human in vitro model.

In order to investigate the effects of BRAF on the actin cytoskeleton, we treated HUVECs with thrombin and analyzed the phosphorylation of ERK downstream of BRAF and the phosphorylation of MLC and coflin, downstream of ROCK. In accordance with previous data, thrombin induced ERK phosphorylation, which was significantly decreased in BRAF-depleted cells (Figure 1D, quantification in Figure 1E). This finding is in agreement with the role of BRAF as the main activator of MEK and ERK. We next assessed the activity of ROCK in HUVECs by monitoring the phosphorylation of MLC and coflin upon thrombin treatment in control and BRAF-depleted cells. In control cells, thrombin treatment increased MLC (Figure 1D, quantification in Figure 1F) and slightly increased coflin phosphorylation (Figure 1D, quantification in Figure 1G), consistent with an increased ROCKII activity upon thrombin treatment. Interestingly, we found that in BRAF-deficient cells MLC was phosphorylated less (2-fold difference) compared to control cells (Figure 1D, quantification in Figure 1F), while the basal level of phosphorylated coflin (p-cofilin) was slightly but significantly (1.5-fold) increased upon BRAF depletion (Figure 1D, quantification in Figure 1G).

Taken together, in BRAF-depleted human endothelial cells both ERK and MLC phosphorylation are decreased, and the basal level of p-cofilin is slightly increased. The latter finding is surprising, because both in mouse BRAF-knockout cells and in fibroblasts the activity of ROCKII on coflin is diminished, even though the underlying mechanisms are different. Therefore, the question arises whether the same (patho)physiological effect in mouse and human cells is realized through different molecular mechanisms. In order to answer this question, we studied the reorganization of the actin cytoskeleton, as well as MLC and coflin localization in thrombin-treated control and BRAF-depleted cells.

3.2 | BRAF-depleted cells have a defect in stress fiber formation and show a peripheral pMLC ring upon thrombin treatment

In control cells, thrombin treatment induced the reorganization of actin filaments (Figure 2A,D). A peripheral actin ring dominated 2 min after stimulation (Figure S1). Subsequently, actin formed thinner stress fibers, and thicker ones were assembled 15 min following treatment. BRAF-deficient HUVECs showed a thicker peripheral actin ring (Figure 2A) compared to control cells, as we previously observed upon BRAF depletion in human, and in mouse endothelial cells. In addition, actin did not form stress fibers in the cell center upon thrombin treatment (Figure 2D). Next, we investigated how thrombin regulates the localization of the phosphorylated form of MLC (pMLC) and how BRAF ablation modulates it. In control cells, the amount of pMLC was increased both in the cell periphery and in the cell center at early time points (Figure S1). pMLC decorated the peripheral actin ring 2 min after thrombin treatment (Figure S1), and later it was also found along the actin fibers in the cell center (Figure 2D–F). In siBRAF cells, pMLC was enriched only in the periphery 2 min after thrombin treatment and did not appear in the cell center even at later time points (Figure S1 and Figure 2E,F). Our immunoblot data show less thrombin-induced pMLC in siBRAF cells compared to that of control cells (Figure 1D,F) upon stimulation; as pMLC is localized exclusively in the cell periphery in BRAF-depleted cells, this decrease is due to the lack of pMLC in the cell center.

In order to determine whether in human cells, the observed increase in peripheral pMLC staining upon BRAF depletion is ROCK-dependent, we treated both control and siBRAF cells with the ROCK-specific inhibitor Y27632 and analyzed pMLC localization after thrombin treatment (Figure 3 and Figure S2). In control cells, Y27632 completely abrogated stress fibers and reduced pMLC staining in the cell center (Figure 3A–C). This is in agreement with the role of ROCK in stress fiber formation. In siBRAF cells, the peripheral pMLC staining was decreased by Y27632, pointing to increased ROCK activity at this location (Figure 3D–F).

Our next question was whether ROCK activity in the cell periphery might be regulated by BRAF through the MEK/ERK module. BRAF has been shown to regulate the activity of ROCK and stress fiber formation in fibroblasts through the MEK/ERK pathway. Stress fiber formation is also prevented upon MEK inhibition in epithelial cells and in bovine aortic endothelial cells. In order to answer the question whether MEK inhibition can phenocopy the
effect of BRAF ablation on the cytoskeleton, we pre-treated control HUVECs with the MEK/ERK inhibitor U0126 and stimulated the cells with thrombin. U0126 prevented stress fiber formation, and a thicker peripheral actin ring was observed compared to control (DMSO-treated) cells (Figure 3G–I and Figure S3). The inhibitor also increased the peripheral localization of pMLC slightly, but to a lesser extent than in BRAF-ablated cells. In addition, U0126 treatment, like BRAF depletion, decreased the phosphorylation of MLC upon thrombin treatment (western blot, Figure S3D).

Taken together, thrombin stimulation increased the amount of pMLC in the cell periphery, both in control and in BRAF-depleted cells at early time points. In control cells, actin reorganized and formed stress fibers at later time points, but this was prevented in the absence...
FIGURE 2  BRAF-depleted cells have a defect in stress fiber formation and show a peripheral pMLC ring upon thrombin treatment. Effect of BRAF knockdown was analyzed on the localization of actin (A and D) and pMLC (B and E) upon 5 min of thrombin treatment. Panels C and F show the merged images of actin (cyan), pMLC (red) and VE-cadherin (gray) stainings. White arrows indicate pMLC-decorated actin fibers after thrombin treatment. Representative images from three independent experiments are shown. Scale bar denotes 25 μm. Figure S1 also show the same immunofluorescence images of HUVEC cells treated with thrombin for two and 15 min for comparison.

FIGURE 3  The pMLC ring in the cell periphery of BRAF-depleted cells is ROCK-dependent, but cannot be completely phenocopied by MEK/ERK inhibition. The effect of the ROCK-specific inhibitor, Y27632 (panels A-F) or the MEK-specific inhibitor, U0126 (panels G-I) was analyzed on the localization of actin (A, D and G) and pMLC (B, E and H) upon thrombin treatment (15 min) both in siControl (A-C and G-I) and siBRAF (D-F) cells. Panels C, F and I show the merged images of actin (cyan), pMLC (red) and VE-cadherin (gray) stainings. Representative images from two independent experiments are shown. Scale bar denotes 25 μm. Both inhibitors were used at 10 μM concentration for 1 h. Figures S2 and S3 also show in the absence of thrombin the same immunofluorescence images of Y27632-treated and U0126-treated HUVECs, respectively.
of BRAF. The observed pMLC ring in the cell periphery of BRAF-depleted cells was ROCK-dependent and it could not be completely phenocopied by MEK/ERK inhibition.

3.3 | BRAF knockdown decreases the amount of peripheral coflin

The western blot data in Figure 1D showed a slightly increased basal coflin phosphorylation in siBRAF cells. Our question was whether this increased phosphorylation is also connected to an increased peripheral localization or activity of ROCK. In order to answer this question, we fixed and stained thrombin-treated HUVEC monolayers with antibodies against coflin (Figure S4) or p-cofilin (Figure S5). Comparison of non-treated siControl and siBRAF cells is shown for coflin and p-cofilin in Figure 4A,B and Figure 4D,E, respectively. We found that coflin staining was homogenous in control cells and did not change significantly upon thrombin stimulation (Figure S4). Interestingly, in siBRAF cells coflin staining was faint in the cell periphery and did not change over time. Quantification of fluorescence intensities of coflin in the periphery (Ip) and in the cell center (Ic) showed that BRAF depletion decreased the ratio Ip/Ic from 0.85–0.91 in control cells to 0.63–0.68 (Figure 4C). In control cells the amount of peripheral coflin decreased over time, but the difference was not significant. The p-cofilin staining pattern was in good agreement with the coflin staining pattern (Ip/Ic ratio 0.88–0.96 in control HUVEC, 0.59–0.72 in BRAF-depleted cells; Figure 4F).

To determine whether the activity of ROCK was necessary for the reduced coflin localization in the periphery of siBRAF cells, we treated siBRAF cells with the ROCK inhibitor Y27632. The results show that coflin was re-localized to the cell periphery of BRAF-deficient cells upon Y27632 treatment (Figure 5A,B), while in control cells the localization of coflin was unchanged (Figure S6A,C). Based on the quantification of coflin fluorescence intensities, in DMSO-treated siBRAF cells the Ip/Ic ratio was 0.72–0.76, while in the presence of Y27632 it increased to 0.91–0.94 (Figure 5C). Very similar Ip/Ic ratios were obtained for BRAF-proficient control cells (Ip/Ic was 0.85–0.91, Figure 4C). Thus, the inhibition of ROCK activity rescued the coflin phenotype observed in siBRAF cells.

In keratinocytes, pharmacological inhibition of the MEK/ERK pathway was shown to induce coflin phosphorylation.33 Therefore, we treated thrombin-stimulated control cells with the MEK inhibitor U0126 and monitored coflin phosphorylation. Our results showed that there were no detectable changes in the level of coflin phosphorylation upon U0126 treatment (Figure S3D). We also analyzed coflin localization after U0126 treatment but did not detect any difference between control and U0126 treated cells (Figure 5D–F and Figure S7).

Taken together, we found that the actin depolymerizing factor coflin is excluded from the cell periphery in siBRAF cells, but not in control cells. This phenotype can be reverted by a ROCK-specific inhibitor Y27632, and it cannot be phenocopied by the MEK inhibitor U0126 in control cells. The data imply that ROCK activity is necessary for the removal of coflin from the cell periphery in siBRAF cells, and that MEK activity does not have any role in coflin regulation upon thrombin treatment in HUVECs.

3.4 | BRAF increases endothelial cell stiffness upon thrombin treatment

To determine whether BRAF affects endothelial cell stiffness, we knocked down BRAF by using shRNA and determined the elasticity of fixed and live cells with atomic force microscopy (AFM). Figure 6A shows the experimental arrangement of the AFM combined with an epifluorescence microscope which was used to identify the EGFP-expressing cells co-expressing either control or BRAF shRNAs. BRAF-knockdown cells were identified by their EGFP expression as described in Hollósi et al.23 Figure 6B illustrates the phase-contrast image of the fixed monolayer, the fluorescence image of the same monolayer, as well as the high-resolution AFM deflection image. The effect of thrombin was investigated on fixed cells, since with our AFM setup there is no possibility to inject thrombin after setting up the system, therefore on the short timescale of thrombin treatment we cannot produce significant amount of data to analyze the effect of thrombin. Cells stimulated with thrombin for different periods of time up to 15 min were fixed and elasticity measurements were carried out in the cell center and in the periphery of individual cells located within a monolayer. The elastic modulus, which is directly proportional to stiffness, was determined for each indentation curve (see Materials and Methods section for details) and examples of its distribution are shown as histograms in Figure S8. Pairwise comparisons illustrate how thrombin stimulation changed the distribution of elastic modulus in the center and in the periphery of control and BRAF-depleted cells. In shControl cells thrombin stimulation induced a shift of the elastic modulus distribution to higher values in the cell center (Figure S8A) and to slightly lower values in the periphery (Figure S8B). In contrast, in shBRAF cells elastic modulus distribution did not change with thrombin treatment in the cell center (Figure S8C) and even slightly shifted to lower values in the cell periphery (Figure S8D). In live
cells, the elastic modulus distribution was very similar for shControl and shBRAF cells without thrombin treatment, both comparing the cell center (Figure S8E) and the cell periphery (Figure S8F).

We then compared the maximum values of the elastic modulus for fixed cells calculated from each histogram for all time points of thrombin treatment (Figure 6C,D). We did not detect any significant difference between the elasticity measured in the cell center and in the periphery of control or shBRAF cells without thrombin treatment (Figure 6C,D). In control cells, there was a decrease in peripheral stiffness at early time point (2 min after thrombin treatment), which returned to the unstimulated level at later time points (Figure 6D). This was followed by an increase in stiffness in the cell center five and 15 min after thrombin stimulation (Figure 6C), the time when stress fibers appeared. shBRAF cells showed a significant decrease in the elastic modulus 2 min after thrombin stimulation in the periphery, but unlike control cells these did not return to normal throughout the observation period. In line with the lack of stress fiber formation shown in Figure 2D,F, there was no significant change in the stiffness of shBRAF cells throughout the whole stimulation in the cell center. Interestingly, in untreated live cells we
found that the cell periphery is stiffer than the cell center (Figure 6E). This stiffness difference was also observed for live bovine aortic endothelial cells, live non-treated and live TNF-α-treated HUVECs. However, no difference was found between the stiffness of the cell center and the periphery of fixed human pulmonary artery endothelial cells in the absence of any stimuli, similar to the stiffness of fixed HUVECs calculated here. These data indicate that fixation differentially affects the stiffness of the central and peripheral regions. It is also known that fixation increases

Figure 5: Cofilin is relocalized to the periphery of siBRAF cells upon Y27632 treatment. The effect of the ROCK-specific inhibitor, Y27632 (10 μM, 1 h) was analyzed on the localization of cofilin (A and B) in siBRAF cells without thrombin treatment. The effect of the MEK-specific inhibitor, U0126 was analyzed on the localization of cofilin (D and E) upon thrombin treatment (15 min) in control cells. Panels B and E besides cofilin staining (gray) also show the plasma membrane (PECAM, red) and the nuclei (Hoechst, blue). Representative images from two independent experiments are shown. Scale bar denotes 25 μm. Quantifications of fluorescence intensity of cofilin shown as a ratio of peripheral (Ip) and central (Ic) intensities are presented for Y27632 (C) and U0126 (F) treatments. * denotes p < .05. Immunofluorescence images of thrombin- and Y27632-treated siControl and siBRAF as well as U0126-treated siControl cells are shown in Figures S6 and S7.
and indeed, our experimental data show that fixation increased endothelial stiffness 25-times in the cell center and 15-times in the cell periphery. Importantly, BRAF depletion did not affect the stiffness of either the cell center or the cell periphery in the absence of thrombin.

Taken together, the elasticity of fixed shBRAF cells was very similar to that of control cells both in the cell center and in the cell periphery in the absence of thrombin and 2 min after thrombin treatment. However, at 5 min BRAF-depleted cells did not show any stiffness changes either in the cell center or in the cell periphery. The increased stiffening observed in the cell periphery and later in the cell center of BRAF-proficient cells correlates with the appearance of stress fibers.

**FIGURE 6** BRAF increases endothelial cell stiffness upon thrombin treatment. Panel A shows the AFM setup combined with an epifluorescence microscope to identify the EGFP-expressing cells co-expressing either control or BRAF shRNAs. Panel B shows an example of the phase-contrast image of the chemically fixed monolayer (showing the AFM cantilever, upper left panel) together with the fluorescence image (lower left panel) of the same monolayer, as well as its high-resolution AFM deflection image (right panel). Boxed region in the phase-contrast and the fluorescence image corresponds to the AFM deflection image, and the red # indicate the same cell within the monolayer in all three images. Elastic moduli of shControl and shBRAF cells calculated from the indentation curves measured either in the cell center (C) or in the cell periphery (D) of fixed cells are plotted for different durations of thrombin treatment. At least 30 cells were analyzed, and 100 indentation curves were recorded for each cell. The results shown were from three independent experiments and were carried out with two different LOTs of HUVECs. Elastic moduli of live shControl and shBRAF cells are compared in (E) for the cell center and the periphery without thrombin treatment and the data were from two independent experiments (two different LOTs of HUVECs). At least 20 live cells were analyzed. * denotes $p < .05$. Scale bar denotes 50 μm. Panel F shows the schematic representation of changes in actin reorganization, pMLC localization and stiffness during the time course of thrombin treatment in control and shBRAF cells.
DISCUSSION

Actin cytoskeletal changes adjust the stiffness of the endothelium and cancer cells like leukocytes during inflammation might sense changes in stiffness to find an optimal place, called hotspot, for extravasation. Therefore, the correlation among permeability, actin (re)organization and regional stiffness changes upon perturbation of the cellular system might help to understand the regulation of the barrier function of the endothelium as well as tumor cell extravasation. Thrombin-induced actin cytoskeletal changes were shown to be important for BRAF V600E mutated melanoma transmigration potential, as the presence of V600E mutation correlates with an increased thrombin production, and the extent of thrombin production correlates with the extravasation efficiency of these cells. Recently, we found that ablation of BRAF in the endothelium makes the endothelial monolayer less permissive in response to thrombin and impairs melanoma metastasis formation in in vitro transmigration assay. We now show that this phenotype can also be observed in human endothelial cells, and that it is linked to a defect in stress fiber formation upon BRAF depletion. We show here that in the absence of BRAF a pMLC ring decorates the peripheral actin, and this depends on the activity of both ROCK and MEK. The increased amount of peripheral pMLC in BRAF-depleted cells might contribute to the stabilization of the thicker peripheral actin ring. The actin depolymerization factor cofilin is depleted from the periphery of siBRAF cells, and this can be rescued by ROCK, but not by MEK inhibition. Several molecular mechanisms could potentially be behind this phenomenon. Upon BRAF ablation, elevated ROCK activity in the cell periphery might increase the local amount of pMLC, and myosin as a competitor of cofilin for F-actin binding might enhance the dissociation of cofilin from peripheral actin. In support of this hypothesis, cofilin RNAi resulted in a pMLC ring formation in HeLa cells, where in addition to F-actin depolymerization and severing cofilin was shown to be involved in a myosin-cofilin competition. Another possible mechanism would be that ROCK phosphorylates cofilin in the cell periphery, promoting dissociation from actin. Indeed, we found an elevated level of basal p-cofilin in the absence of BRAF, that reflects an increased ROCK activity towards LIMK and cofilin. Either way, a more stable actin ring is formed in BRAF-depleted cells, which might be explained by the lack of cofilin depolymerizing/severing activity, and the presence of pMLC in the cell periphery. On the other hand, BRAF might also regulate the activity of proteins responsible for actin polymerization in the cell periphery such as Arp2/3 or mDia, since mDia was shown to protect the Arp2/3-polymerized, branched actin network from debranching, by depleting cofilin through enhancing filament turnover.

In untreated cells, the thicker peripheral actin ring formed upon BRAF depletion does not result in increased peripheral stiffness. This observation is valid for both live and fixed cells. The effects of thrombin were analyzed on fixed cells. Interestingly, early thrombin treatment decreases peripheral stiffness independently of BRAF. This correlates with an increased amount of pMLC on peripheral actin of both BRAF-deficient and BRAF-proficient cells. The peripheral increase in pMLC might be a prerequisite for stress fiber formation in control cells, as proposed by Hirano et al. However, our observation that this happens in the absence of BRAF suggests rather that the decrease in stiffness in concomitance with an increased localization of pMLC might be required but it is not sufficient for stress fiber formation (Figure 6F). At later time points in control cells the stiffness increases both in the cell center and in the cell periphery, and this is accompanied by pMLC-decorated stress fiber formation. Since this step is missing in the absence of BRAF, the increase in peripheral stiffness of control cells might reflect the actin reorganization in the cell periphery required for stress fiber formation in the cell center. It is interesting that the increase of pMLC-decorated actin in the cell periphery of both control and BRAF-depleted cells results in a decreased stiffness, while the formation of pMLC-decorated central actin fibers correlates with an increased stiffness. One explanation for this apparent contradiction could be that the increase of pMLC in the cell periphery is required for the reorganization of actin, but this happens later and only in BRAF-proficient cells; indeed, this finding is in line with the increase in peripheral stiffness observed after 2–5 min of thrombin stimulation. BRAF depletion prevents stiffness increase in the cell center upon thrombin stimulation, which can be explained by the lack of stress fiber formation in the absence of BRAF. The absence of peripheral cofilin and a concomitant increase of peripheral pMLC are not sufficient for changing the stiffness of the cell periphery. Peripheral cofilin might be required rather for the reorganization of peripheral actin upon thrombin treatment, which might limit the localization of pMLC on peripheral actin. We propose that a balance between the binding of cofilin and myosin to F-actin in the cell periphery, which is regulated by the activity of ROCK, determines the dynamics of actin reorganization, which ultimately drives or prevents stress fiber formation.

Thus, our data underscore the importance of the dynamic actin reorganization capacity in the cell periphery, which is essential for intercellular gap formation. Indeed, it was also found that traction force instability, rather than the magnitude of the forces, correlates with intercellular gap formation, and these force fluctuations
localized close to cell–cell junctions. All these forces align with the F-actin cytoskeleton, independently of their localization. In addition, VE-cadherin-containing junctions have a major role in balancing tension between adjacent cells, since almost half of the overall force impinges on cell–cell junctions. Therefore, subtle regulation of actin dynamics in the cell periphery determines the extent of opening/closing of the endothelial barrier. Interestingly, the cortical actin cytoskeleton was shown to contribute to the formation of filopodia-like protrusions at intercellular gaps, playing a role in gap closure. Indeed, during leukocyte transmigration increased RhoA activity around the pore limits vascular leakage, and in our model in BRAF-depleted cells an increased ROCK activity might result in an increase in endothelial barrier function. Since BRAF regulates the localization of ROCK via RAF1, recently described BRAF inhibitors disrupting heterodimer formation between BRAF and RAF1, such as PLX8394 might mimic BRAF depletion. The application of such inhibitors might ultimately strengthen the endothelial barrier function and would be potentially efficient to reduce metastasis.

AUTHOR CONTRIBUTIONS
Anna Hollósi, Katalin Pászty and Andrea Varga designed the experiments. Anna Hollósi, Katalin Pászty, Bálint Levente Bunta, Márta Lídia Debreczeni, Tamás Bozó and Andrea Varga performed experiments. Anna Hollósi, Katalin Pászty, Bálint Levente Bunta, Márta Lídia Debreczeni and Andrea Varga analyzed data. Anna Hollósi, Katalin Pászty and Andrea Varga wrote the manuscript. Manuela Baccarini provided input into research design and data analysis and helped write the article. László Cervenak and Kellermayer Miklós contributed with research tools, with valuable comments to the experimental design and to the manuscript. All authors contributed feedback to the manuscript.

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DISCLOSURES
The authors declare no competing interests.

CODE AVAILABILITY
This study did not generate any code.

DATA AVAILABILITY STATEMENT
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL
Not applicable.

MATERIALS AVAILABILITY
All unique/stable reagents generated in this study are available from the lead contact, Dr. Andrea Varga, upon request.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this 
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