Role of the small GTPase Rap1 for integrin activity regulation
in endothelial cells and angiogenesis

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Running title: Role of Rap1a, Rap1b and RAPL in angiogenesis

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Abstract

Rap1, a small GTPase, attracted attention because of its involvement in several aspects of cell adhesion, including integrin- and cadherin-mediated adhesion. Yet, the role of Rap1 genes and of Rap1 effectors for angiogenesis has not been investigated so far. HUVEC express Rap1a and Rap1b mRNA. In order to determine the contribution of Rap1 activity for angiogenesis, we overexpressed Rap1GAP1, a GTPase activating protein, which specifically inhibits Rap1 activity. Overexpression of Rap1GAP1 significantly blocked the angiogenic sprouting and the tube forming activity of HUVEC as well as migration and integrin-dependent adhesion. Additionally, silencing of either Rap1a, Rap1b or both significantly blocked HUVEC sprouting under basal and bFGF-stimulated conditions and reduced HUVEC migration and integrin-dependent adhesion. In line with these results, we found that Rap1a and Rap1b are essential for the conformational activation of β1-integrins in endothelial cells. Furthermore, silencing of Rap1a and Rap1b prevented the phosphorylation of tyrosine 397 in FAK and the VEGF-induced Akt1-activation. Rap1a⁻/⁻-deficient and Rap1a⁺/⁻ heterozygote mice displayed reduced neovascularization after hind limb ischemia in comparison to wild-type mice. Moreover, silencing of RAPL significantly blocked the Rap1-induced sprouting of HUVEC suggesting that the angiogenic activity of Rap1 is partly mediated by RAPL. Thus, our data demonstrate a critical role of Rap1 in the regulation of β1-integrin affinity, adhesion and migration in endothelial cells and in postnatal neovascularization.
Introduction

The term angiogenesis refers to the formation of new blood capillaries from preexisting ones\textsuperscript{1,2}. Angiogenesis is implicated in many physiological and pathological conditions, including embryonic development, wound healing, tumor growth, rheumatoid arthritis and proliferative retinopathy\textsuperscript{1,2}. Vascular endothelial growth factor (VEGF) is an essential cytokine for vasculogenesis and angiogenesis\textsuperscript{3}. Through its receptors, which include two distinct tyrosine kinases, VEGF exerts multiple effects on endothelial cells including proliferation, rapid induction of endothelial permeability, promotion of endothelial cell survival, stimulation of migration and induction of gene expression\textsuperscript{3}. Besides VEGF, the fibroblast growth factors (FGFs) are also implicated in angiogenesis\textsuperscript{4}. However, the downstream signaling pathways mediating the angiogenic effects of angiogenic growth factors such as VEGF and FGFs are poorly understood.

Integrins are heterodimeric transmembrane proteins consisting of non-covalent bound \( \alpha \)- and \( \beta \)-subunits mediating cell adhesion to extracellular matrix proteins and bidirectional signaling\textsuperscript{5}. Beyond angiogenic growth factors there is evidence that integrins are implicated in angiogenesis\textsuperscript{6}. Specifically, \( \beta1 \)-integrins were shown to play an important role in angiogenesis\textsuperscript{6}. Moreover, angiogenic factors such as VEGF affect integrin activity and function in endothelial cells\textsuperscript{7}.

Ras-associated protein (Rap) proteins define a family of highly homologous small GTP-binding proteins belonging to the Ras superfamily, which includes five members, Rap1a, Rap1b, Rap2a, Rap2b, and the recently discovered Rap2c, which are grouped into two subfamilies, Rap1 and Rap2, based on their sequence homology\textsuperscript{8-12}. Small GTPases cycle between an inactive GDP-bound conformation and an active GTP-bound conformation. In their active conformation, small GTPases interact with effector proteins, which induce downstream signaling. The GDP/GTP cycle is highly regulated by guanine nucleotide exchange factors (GEF) that induce the release
of the bound GDP to be replaced by the more abundant GTP and by GTPase activating proteins (GAP) that promotes GTP hydrolysis\textsuperscript{13}.

Rap1a and Rap1b were shown to be essential for inside-out integrin activation and for integrin-dependent cell-matrix and cell-cell adhesion of various cell types such as leukocytes, platelets, ovarian carcinoma cells, fibroblasts and progenitor cells\textsuperscript{10,11,14-24}. In addition, while this study was under preparation for submission, Chrzanowska-Wodnicka et al have demonstrated that Rap1b is involved in angiogenesis\textsuperscript{25}. Moreover, inactivation of Rap1 by overexpression of Rap1GAPII inhibited wound healing in endothelial cells\textsuperscript{26}. However, the role of Rap1 for integrin activity regulation, integrin-dependent adhesion and angiogenic sprouting of endothelial cells has not been investigated so far. Furthermore, the role of Rap1a for the ischemia-induced neovascularization is unclear.

Active (GTP-bound) Rap1a was shown to associate with effector proteins such as RAPL and RIAM, thus mediating the activation of integrins and integrin-dependent adhesion in leukocytes\textsuperscript{27,28}. Beyond integrin-dependent adhesion, Rap1 regulates junctional adhesion, thereby, modulating permeability of endothelial cell monolayers\textsuperscript{29-33}.

Although Rap1 was shown to be important for the regulation of several physiological processes, little information is available about the role of Rap1 in integrin signaling in endothelial cells and angiogenesis. In the present study, we demonstrated that both Rap1a and Rap1b play a key role in integrin-dependent angiogenic functions such as sprouting, tube formation, migration and adhesion of endothelial cells \textit{in vitro}. In line with these results, Rap1a heterozygote and Rap1a–deficient mice displayed a decreased angiogenic response in the matrigel assay and in the hind limb ischemia model in comparison to wild type mice. Moreover, we provide insights into the Rap1 down-stream signaling pathways and demonstrate that RAPL partly mediates the angiogenic effects of Rap1.
Materials and Methods

Cells
Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Germany) and cultured in endothelial basal medium (EBM) supplemented with 1 µg/mL hydrocortisone, 12 µg/mL bovine brain extract, 50 µg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor and 10% fetal calf serum until the third passage.

Plasmid constructs and transfection
HUVECs (3.5 × 10^5 cells/6-cm well) were grown to 60–70% confluence and then transfected with 3 µg of plasmids and 20 µL Superfect (Qiagen, Germany), resulting in a transfection efficiency of about 50% as previously described34,35. The Rap1GAP1 (pcDNA-Flag-Rap1GAP1) was kindly provided by Dr. P.J. Stork (Vollum Insitute, Oregon Health and Science University, Portland, Oregon), the plasmids EGFP-Rap1a, the constitutive active mutant EGFP-Rap1aV12 and GFP-RBD_RalGDS were kindly provided by Dr. M. R. Philips (New York University, New York)36. We subcloned Rap1a and Rap1aV12 from the EGFP-Rap1a- and EGFP-Rap1aV12-vectors in the pcDNA3.1-His vector.

RNA interference
To silence Rap1 and RAPL expression, we performed transfection of siRNA duplex using GeneTrans II (MoBiTec, Germany). Rap1a, Rap1b and RAPL siRNAs were synthesized by Eurogentec (Germany). The Rap1a target sequence was 5'-GCAAGACAGTGGTGTAACT-3' (Rap1a siRNA I), the Rap1b target sequence was 5'-GTCTGCTTTGACTGTACAA-3' (Rap1b siRNA I). The human RAPL siRNA target sequence was 5'-CTGGAAGACTGCTTCTTCA-3' (RAPL siRNA I). A non-related, scrambled siRNA was used as a control. Additional sequences are available upon request.
Western blot analysis

For Western blot analysis, HUVECs were lysed with 100 μL lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol Na₃VO₄, 1μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol NaF) for 15 minutes on ice. After centrifugation for 15 minutes at 14,000 RPM (4°C), the protein content of the samples was determined according to the Bradford method. Proteins were loaded onto SDS-polyacrylamide gels and blotted onto PVDF membranes (Millipore, Germany). Western blots were performed by using antibodies directed against Rap1 (1/1000, Upstate, Germany), phospho-tyrosine 397 FAK (1/1000, Biosource, Germany), FAK (1/1000, BD bioscience, Germany) phospho-Erk1/2 (1/1000, Cell Signaling, Germany), Erk1/2 (1/1000, Cell Signaling, Germany) FLAG (1/1000, Sigma, Germany) and α-tubulin (1/1000, Dianova, Germany). Enhanced chemiluminescence was performed according to the manufacturer’s instructions (Amersham Biosciences, Germany). Densitometry was performed where indicated for the quantification of the Western blots.

Reverse-transcription PCR

Total RNA was isolated using the RNAeasy Mini Kit (Qiagen, Germany). Afterwards, 1 µg of RNA from each sample was reverse transcribed into cDNA and subjected to conventional PCR. Primer sequences for PCR were: Rap1a 5’-CGATTGCCAACAGTGTATGCTCG-3’ and 5’-ACACCACCTGTCTTGTGCTTAATTCTG-3’; Rap1b 5’-TTTTATTCCATCACAGCAGATCC-3’ and 5’-TTTCTGTTAATTTGCGCMEACTAG-3’ and RAPL 5’-CCTGGACGAGAAGACAGTAGCC-3’ and 5’-CAACCATGAACCTTTCTTGAGCAGCC-3’.

Tube Formation Assay
Two hundred microliters of Matrigel Basement Membrane Matrix (BD Biosciences, Germany) were coated for two hours at 37°C within a 12-well plate (Greiner, Germany). Transfected HUVEC were detached by trypsinization, and after neutralization of trypsin, cells were resuspended in EBM containing 10% FCS. Then, HUVECs \(1 \times 10^5\) were seeded in 1 mL of EBM (10% FCS) on the Matrigel Basement Membrane Matrix. Tube length was quantified after 24 h by measuring the cumulative tube length in five random microscopic fields with a computer-assisted microscope (Axiovert 100, Carl Zeiss, Jena, Germany) using the software Axiovision 4.5 (Carl Zeiss, Jena, Germany).

**Spheroid-based angiogenic assay**

Endothelial-cell spheroids of defined cell numbers were generated as described previously \(^3\)\(^7\). In brief, HUVEC were suspended in culture medium containing 0.2% (wt/vol) carboxymethylcellulose (Sigma, Germany) and seeded in round-bottom 96-well plates which do not support cell adhesion (Greiner, Frickenhausen, Germany). Under these conditions, all suspended cells contribute to the formation of a single spheroid per well of defined size and cell number (400 cells/spheroid). Spheroids were generated overnight and then embedded into rat collagen I (BD Bioscience, Germany). The spheroid-containing collagen was rapidly transferred into prewarmed 24-well plates and allowed to polymerize (for 30 minutes). Then, 100 µL EBM with or without human basic fibroblast growth factor (bFGF, 50 ng/mL, Peprotech, Germany) was added into the wells. In the experiments using siRNA-silencing, HUVEC were transfected 48 h before the formation of the spheroids with the indicated siRNAs. For the experiments with the neutralizing β1-antibodies, murine IgG1 (30µg/mL, Ancell, Germany) or anti-β1-integrin (clone 6S6, 30µg/mL, Chemicon, Germany) was added to the collagen gel. After 24 hours, pictures were taken using an Axiovert 100 microscope and a Plan-NEOFLUAR 10x objective. **In vitro** capillary sprouting was quantified by measuring the cumulative length of sprouts per each
spheroid using AxioVision Rel 4.5 digital imaging software (Carl Zeiss, Jena, Germany). The mean cumulative sprout length per spheroid was calculated after evaluation of 10-15 spheroids / condition.

**Cell-matrix adhesion**

Ninety-six-well plates were coated overnight at 4 °C with 1µg/mL soluble recombinant human collagen I (Chemicon, Mannheim, Germany) or 2.5µg/mL human fibronectin (Roche, Mannheim, Germany) or 2.5µg/mL human vitronectin (Chemicon, Mannheim, Germany) in PBS and then blocked for one hour at room temperature with 3 % (w/v) heat-inactivated (2 h, 56 °C) bovine serum albumin (BSA, Sigma, Germany). HUVEC were stained with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM) and after detachment with trypsin were resuspended in EBM containing 0.05 % BSA. Then, cells were seeded at 50,000 cells/well in 100 µL in the wells for 60 min at 37 °C. After removal of non-adhering cells by washing with warm EBM, adherent cells were quantified in triplicates with a fluorescence plate reader (Synergy HT, Biotek, Germany).

**HUVEC migration**

Transwell membranes (8 µm; Costar, Germany) were coated on both sides with fibronectin (2.5 µg/mL; Roche, Mannheim, Germany) or human collagen type I (1 µg/mL; Chemicon, Schwalbach, Germany) overnight at 4°C. HUVEC were detached by trypsinization, and after neutralization of trypsin, cells were resuspended in serum-free EBM containing 0.05% BSA. Then, HUVEC (50,000 cells/well) were incubated in the upper chamber at 37°C in 5% CO₂ and allowed to migrate for 5 hours toward the lower chamber in the presence or absence of VEGF 50 ng/mL (Peprotech, London, UK). Cells remaining on the upper surface of the transwells membranes were mechanically removed and cells that had migrated to the lower surface were
fixed with 4% formaldehyde. For quantification cell nuclei were stained with DAPI. Cells migrated into the lower chamber were counted in 5 random microscopic fields using a fluorescence microscope (Axiovert 100, Carl Zeiss, Jena, Germany).

**Activation of Rap1 in migrating HUVEC**

HUVEC were plated on glass slides coated with 2.5µg/mL fibronectin (Roche, Mannheim, Germany). After 36 h, HUVEC were transfected with GFP-RBD<sub>RalGDS</sub>. 6 hours after transfection, a scratch wound was created with a cell scraper. After 3 hours, cells were fixed in 3% formaldehyde in PBS for 10 min at room temperature, and DAPI nuclear staining was performed. Cells were analyzed with a confocal microscope for localization of the active Rap1 (LSM510, Carl Zeiss, Jena, Germany).

**Total and Phospho-Akt1-ELISA**

The experiments were performed 48 h after transfection with the respective siRNAs. Transfected HUVEC were serum-starved for 2 hours in EBM (0% FCS) and then stimulated where indicated with VEGF (50 ng/mL) for 5 min. After lysis with the Akt-ELISA lysis buffer, Akt-ELISA assays were performed (total Akt1-ELISA and phospho-Akt1 (ser473)-ELISA) using a commercial Akt1-ELISA Kit (Cell Signaling Technology, Frankfurt, Germany). Absorbances were measured with a plate reader (Synergy HT, Biotek, Germany).

**Immunofluorescence staining**

The method for the immunofluorescence staining is described in the Online Supplemental material (Supplemental methods and materials Page 1).

**Animal experiments**
All the animal experiments were approved from the Regional Board of Land Hessen, Darmstadt Germany. The animal experiments are described in the Online Supplemental material (Supplemental methods and materials Page 1).

**Statistical analysis**

Continuous variables are expressed as mean ± SEM. Comparisons between groups were analyzed by t-test (two-sided) or ANOVA (post hoc test: LSD) for experiments with more than two subgroups (SPSS software). *P* values <0.05 were considered as statistically significant.
Results

Expression of Rap1a and Rap1b in human endothelial cells

In order to characterize the expression of Rap1a and Rap1b in human endothelial cells, we performed RT-PCR. HUVEC expressed both Rap1a and Rap1b mRNA as assessed by RT-PCR (Supplemental Figure S1A). Moreover, analysis of a mRNA expression microarray revealed that Rap1a and Rap1b are similarly expressed in HUVEC and in human microvascular endothelial cells (HMVEC) (supplemental Figure S1A and Figure S1B). We confirmed the expression of Rap1 in HUVEC by Western blot (data not shown). Since VEGF and bFGF are relevant angiogenic growth factors, we investigated their effects on Rap1 activation by using Rap1 activity assays. Both, VEGF and bFGF, rapidly increased the active GTP-bound Rap1 reaching a maximum between 2 and 5 min after stimulation (supplemental Figures S1C and S1D). This activation was reversible within 15 min (supplemental figure S1D). All microarray data has been deposited with Gene Expression Omnibus (GEO) under accession number GSE2040.

Role of Rap1 activity for angiogenic sprouting and tube formation

Since angiogenic growth factors rapidly increase Rap1 activity, we next investigated, whether Rap1 activity is required for angiogenic sprouting and tube formation of endothelial cells in vitro. Rap1GAP1 is a GTPase-activating protein, which specifically inhibits Rap1a and Rap1b activity. To investigate the role of Rap1 activity in angiogenesis, we employed a 3-dimensional spheroidal system of endothelial differentiation and in vitro angiogenic sprouting. Endothelial cell spheroids were embedded in collagen gels, and outgrowth of capillary-like structures was assessed. Overexpression of Rap1GAP1 significantly blocked the basal and bFGF-induced angiogenic sprouting of HUVEC in this spheroidal culture system (Figure 1A and 1B). Consistent with these data, overexpression of Rap1GAP1 led to a significant impairment of
tube/network-forming activity of HUVEC in another angiogenic assay, the matrigel assay (Figure 1C). Overexpression of Rap1a or a constitutively active Rap1aV12 mutant significantly increased the angiogenic sprouting of HUVEC in comparison to mock-transfected cells (Figure 1D). Moreover, the Rap1aV12-induced sprouting of HUVEC was inhibited by a neutralizing β1-integrin antibody (Figure 1E). In addition, the neutralizing β1-integrin antibody also inhibited bFGF-induced angiogenic sprouting of HUVEC (Supplemental Figure S4B). In conclusion, these data demonstrate that Rap1 activity is required and sufficient to promote in vitro angiogenesis in a β1-integrin-dependent manner.

Role of Rap1a and Rap1b for angiogenic sprouting

Having demonstrated that Rap1 activity is essential for the angiogenic sprouting of endothelial cells in vitro, we next sought to separately study the role of endogenous Rap1a and Rap1b for angiogenesis. For this purpose, we designed small interfering RNA (siRNA) sequences specifically targeting Rap1a and Rap1b. RT-PCR and Western blot confirmed the efficient and specific suppression of Rap1a and Rap1b by the respective siRNA oligonucleotides (Figure 2A and supplemental Figure S2). Interestingly, silencing of Rap1a or Rap1b by siRNA transfection significantly blocked the angiogenic sprouting of HUVEC under basal conditions and upon stimulation with bFGF (Figure 2B). Simultaneous silencing of Rap1a and Rap1b induced a minor further decrease in sprout formation, which did not achieve statistical significance compared with silencing of the single genes (Figure 2B). As a control for the specificity of this approach, a second Rap1a- and Rap1b-specific siRNA was generated and gave identical results in terms of angiogenic sprouting (data not shown). Remarkably, silencing of both, Rap1a and Rap1b, did not significantly influence the proliferation rate of HUVEC (data not shown). Taken together, Rap1a and Rap1b are essential for in vitro angiogenesis without affecting proliferation.
Role of Rap1a and Rap1b for endothelial cell migration

Endothelial cell migration is an essential step during angiogenesis. Therefore, we investigated the localization of active (GTP-bound) Rap1 in migrating endothelial cells. For this purpose, we overexpressed a construct containing the Ras-Binding-Domain (RBD) of RalGDS fused with GFP as an indicator of Rap1 activity in HUVEC. Interestingly, active (GTP-bound) Rap1 partly accumulated at the plasma membrane preferentially in the leading front of migrating HUVEC in a scratch wound assay in comparison to non-migrating endothelial cells, which displayed a more random localization of active Rap1 on their cell membrane or localization of active Rap1 to cell-cell contacts (Figure 3A and data not shown). These data suggested that Rap1 activity may be involved in the regulation of endothelial cell migration. To test this hypothesis, we overexpressed Rap1GAP1 in HUVEC and performed migration assays on the matrix proteins collagen and fibronectin. Remarkably, inhibition of Rap1 activity significantly blocked basal and VEGF-stimulated migration of HUVEC on both matrix proteins (Figure 3B). Moreover, silencing of Rap1a and/or Rap1b inhibited the basal and the VEGF-induced migration of HUVEC on fibronectin (Figure 3C) and on collagen (supplemental Figure S3A).

Role of Rap1a and Rap1b for endothelial cell adhesion and for β1-integrin affinity regulation

The regulation of cell adhesion to matrix proteins is essential for cell migration and angiogenesis. Therefore, we studied the role of Rap1 activity for the integrin-dependent endothelial cell adhesion on fibronectin and collagen. Overexpression of Rap1GAP1 significantly reduced the adhesion of HUVEC on extracellular matrix proteins fibronectin and collagen (Figure 4A). In addition, silencing of Rap1a, Rap1b or of Rap1a and Rap1b, significantly blocked the adhesion of HUVEC on the matrix proteins fibronectin (Figure 4B), collagen and vitronectin, which is the major ligand for αVβ3- and αVβ5-integrins (supplemental figures S3B and S3C). Since β1-integrins are mediating cell adhesion to fibronectin and to collagen.
collagen and are essential for the Rap1aV12-induced *in vitro* sprout formation in endothelial cells (Figure 1E), we investigated the role of Rap1 for β1-integrin expression. Interestingly, inhibition of Rap1 activity by overexpression of Rap1GAP1 did not affect the surface expression of β1-integrins in HUVEC (supplemental figure S3D). These data prompted us to investigate the role of Rap1a and Rap1b on integrin affinity, which is promoted by conformational changes of the integrin subunits\(^4\). For this purpose, we performed immunofluorescent staining using an antibody (clone HUTS21) which recognizes the active conformation of β1-integrins. Stimulation of HUVEC with 8-pCPT-cAMP, a specific activator of the guanine nucleotide exchange factor Epac, which specifically increases Rap1 activity, enhanced the staining with HUTS21 in comparison to non-stimulated endothelial cells (Figure 4C). Silencing of Rap1a and Rap1b reduced the active conformation of β1-integrins under basal conditions and upon stimulation with 8-pCPT-cAMP in comparison to scrambled siRNA-transfected HUVEC (Figure 4C), suggesting that active Rap1 is essential for the regulation of β1-integrin affinity in endothelial cells. Taken together, these data indicate that Rap1a and Rap1b promote angiogenesis by mediating β1-integrin affinity regulation, thereby mediating endothelial cell adhesion and migration.

**Role of Rap1a and Rap1b for angiogenic signaling**

Silencing of Rap1a and Rap1b blocked angiogenic sprouting, migration and adhesion of endothelial cells. Therefore, we investigated the role of Rap1a and Rap1b for angiogenic signaling in endothelial cells. Silencing of Rap1a and Rap1b blocked the VEGF-induced phosphorylation of Akt1 on serine 473 in adherent HUVEC as assessed by an Akt-Phospho-ELISA assay (Figure 5A). In addition, knock down of Rap1a and Rap1b reduced the phosphorylation/activation of ERK under basal conditions but had only a slight inhibitory effect on VEGF-induced ERK phosphorylation (Figure 5B). Moreover, silencing of Rap1a and Rap1b
reduced the phosphorylation of FAK on tyrosine 397 (Figure 5C). In conclusion, these data demonstrate that Rap1 is required for appropriate angiogenic signaling in endothelial cells.

Role of Rap1a for in vivo angiogenesis

Next, we studied the role of Rap1a for in vivo angiogenesis. Rap1a+/− mice (in C57Bl/J background) are born with a substantially reduced mendelian ratio: 32.3% : 59.1% : 8.6% ratio of Rap1a+/+, Rap1a+/− and Rap1a−/− mice, respectively (313 mice born from heterozygote mating). We studied the role of the Rap1a in postnatal angiogenesis by using the Rap1a+/− heterozygote mice and the Rap1a-deficient mice (Rap1a−/−). Cells isolated from Rap1a+/− heterozygote mice displayed about 50 % reduction of Rap1a mRNA (data not shown). Moreover, we investigated the role of Rap1a for the ischemia-induced neovascularization using the murine hind limb ischemia model. Strikingly, Rap1a+/− and Rap1a+/− mice displayed a reduced capillary (Figure 6A) and arterioles density (Figure 6B) 14 days after the induction of hind limb ischemia in comparison to wild type mice. In line with these results, the perfusion of ischemic muscles detected by ultrasound was reduced in Rap1a−/− mice in comparison to wild type mice (Figure 6C). In an additional angiogenic model, Rap1a+/− heterozygote mice displayed a significant reduction in capillary formation in a matrigel assay in comparison to wild-type (Rap1a+/+) mice (Figure 6D). In conclusion, Rap1a is essential for postnatal angiogenesis in vivo.

RAPL is a downstream effector of Rap1 mediating angiogenesis

Having demonstrated the importance of Rap1a and Rap1b for angiogenesis, we finally aimed to identify components of the downstream signaling pathway mediating these effects. RAPL is an
effector of Rap1, which associates with activated (GTP-bound) Rap1 and mediates integrin activation in lymphocytes\cite{27,42}. Therefore, we studied the role of RAPL in angiogenesis. HUVEC expressed mRNA for RAPL (Figure 7A). Silencing of RAPL significantly blocked the angiogenic sprouting and migration of HUVEC (Figures 7B and Figure 7C). As a control for the specificity of this approach, a second RAPL-specific siRNA was generated and gave identical results in terms of angiogenic sprouting (Supplemental Figure 4). In order to investigate the role of RAPL as a possible downstream effector of Rap1 in angiogenesis, we overexpressed the constitutively active mutant Rap1aV12 in combination with scrambled or RAPL siRNA. Whereas the Rap1aV12 mutant increased angiogenic sprouting in scrambled siRNA-transfected cells, the silencing of RAPL inhibited the Rap1aV12-induced sprouting (Figure 7D and 7E). However, Rap1aV12 still slightly but significantly increased sprouting formation in HUVEC when co-transfected with RAPL siRNA compared to HUVEC transfected with RAPL siRNA and empty vector, indicating the existence of additional downstream effectors beyond RAPL to mediate angiogenesis (Figure 7E). Taken together, these data demonstrate that RAPL is a downstream mediator of Rap1-induced angiogenesis, but also suggest that additional downstream angiogenic effectors of Rap1 may exist.
Discussion

The present study underscores the relevance of Rap1a and Rap1b for integrin function in endothelial cells and *in vitro* and *in vivo* angiogenesis. Specifically, the data of the present study revealed that: 1) human endothelial cells express the Rap1a and Rap1b genes, 2) angiogenic growth factors can rapidly increase Rap1 activity in endothelial cells, 3) Rap1a and Rap1b are essential for *in vitro* angiogenic sprouting, migration, adhesion and integrin affinity regulation in endothelial cells, 4) Rap1a and Rap1b are essential for the VEGF-induced phosphorylation of Akt in adherent endothelial cells, 5) using the Rap1a heterozygote mice, we demonstrate that Rap1a is essential for the *in vivo* neovascularization, and finally 6) we identified RAPL as an important effector of Rap1 mediating angiogenic sprouting. Thus, the present study provides insights into the role of integrin regulation for angiogenesis in endothelial cells and unravels a new function of Rap1 as key mediator of neovascularization.

In this work, we demonstrated that angiogenic growth factors like VEGF and bFGF rapidly increase Rap1 activity. However, the underlying mechanism is unclear. It is conceivable, that a guanine nucleotide exchange factor (GEF), which potentially acts downstream of the VEGF- and bFGF-receptors could mediate this effect. A possible candidate GEF is C3G, a GEF of Rap1, which mediates signals from receptor tyrosine kinases. Indeed, it was shown that C3G can mediate VEGF- and bFGF-induced signaling in endothelial cells. However, the role of C3G for the bFGF- and VEGF-induced Rap1-activation in endothelial cells is not established. Further studies are required to clarify the mechanism of VEGF- and bFGF-induced Rap1 activation in endothelial cells.

Remarkably, inhibition of Rap1 activity, or knock down of Rap1a or Rap1b blocked the angiogenic sprouting and tube forming activity of endothelial cells. In line with these results, the Rap1a heterozygote mice displayed a reduced angiogenic response in an *in vivo* matrigel plug assay and in the hind limb ischemia model. Interestingly, a recent study showed that the Rap1b-
deficient mice also displayed angiogenic defects\textsuperscript{46}. In addition, generation of knock-out mice for C3G and PDZ-GEF, two GEFs of Rap1, led to defective vascular morphogenesis\textsuperscript{47,48}. Taken together, our data clearly demonstrate that Rap1a and Rap1b are involved in the sprouting of endothelial cells and \textit{in vivo} angiogenesis. However, some of the single Rap1a- and Rap1b-deficient mice are born displaying no vascular defects under normal conditions. The modest phenotype might be explained by a redundant function of Rap1a and Rap1b and it is conceivable that mice lacking the endothelial Rap1a and Rap1b may display a more severe vascular phenotype during embryonic vascular development than the single-deficient mice. Another possibility is that during vascular development additional signaling pathways may compensate for Rap1a and Rap1b. In line with this results, silencing of Rap1a and Rap1b did not completely abolish the angiogenic sprouting, migration and adhesion of endothelial cells suggesting that additional molecular pathways may mediate these angiogenic effects.

Cell migration is an essential angiogenic function of endothelial cells. The role of Rap1 in cell migration is controversially discussed. It was demonstrated that the migration of C3G-deficient fibroblasts was increased in comparison to wild type cells and that expression of active Rap1 blocked this effect\textsuperscript{49}. Similarly, overexpression of Rap1V12 blocked cell motility in carcinoma cells\textsuperscript{50}. However, in other studies inhibition of Rap1 blocked cell motility and activation of Rap1 correlated with increased migratory capacity\textsuperscript{21,24,26,51}. The data of the present study revealed that overexpression of Rap1GAP1 or silencing of Rap1a and/or Rap1b inhibited the integrin-dependent endothelial cell migration on fibronectin and collagen. Furthermore, active Rap1 was localized at the front of migrating endothelial cells. These data clearly demonstrate that Rap1a and Rap1b are essential for endothelial cell migration.

Rap1 was shown to be an essential activator of adhesion in a variety of cells (e.g. platelets, leukocytes, fibroblasts) \textsuperscript{11,52}. However, the role of Rap1 for the interaction of endothelial integrins to extracellular matrix proteins has not been investigated so far. Since endothelial cell migration
on matrix proteins was reduced by inhibition of Rap1, we additionally studied the role of Rap1a and Rap1b on integrin-affinity and integrin-dependent interaction of endothelial cells with extracellular matrix proteins. Strikingly, inhibition of Rap1 activity or knock down of Rap1a and/or Rap1b significantly blocked the β1-integrin-dependent adhesion of endothelial cells to fibronectin and collagen and the αVβ3/αVβ5-integrin-dependent adhesion to vitronectin. This effect was mediated, at least for the β1-integrins, by an inhibitory effect on integrin affinity. Activated integrins were shown to localize in the front of migrating endothelial cells in a Rac1-dependent manner. Since active Rap1 and activated integrins localize at the leading front of migrating endothelial cells and Rap1 can increase and affect the localization of Rac1 activity, it is conceivable that inhibition of Rap1 may affect migration through an inhibitory effect on integrin activity and on the localization of activated integrins.

Interestingly, silencing of Rap1a and Rap1b inhibited the VEGF-induced Akt phosphorylation in adherent endothelial cells. A possible explanation for this effect could be that silencing of Rap1a and Rap1b affects the adhesion-dependent Akt phosphorylation and activity mediated by outside-in integrin signaling. Indeed, it has been demonstrated that integrin-dependent outside-in signaling acts synergistically to receptor tyrosine kinases affecting their downstream signaling. Another possibility is that Rap1 or a Rap1 effector act downstream of the VEGFR2 as essential cofactor for Akt phosphorylation. Independent on the mechanism by which Rap1 interferes with Akt signaling VEGF-induced Akt phosphorylation regulates eNOS, Girdin as well as ACAP1 phosphorylation, all involved in migration, thereby, contributing to the migratory defect in Rap1a- and Rap1b-silenced endothelial cells. Akt may also affect integrin affinity and adhesion. Besides Akt phosphorylation, silencing of Rap1a and Rap1b impaired phosphorylation of FAK on tyrosine residue 397 in endothelial cells. This effect is also indicative of an impaired integrin-dependent outside-in signaling, which may affect endothelial cell motility. Furthermore, silencing of Rap1 and Rap1b affected basal ERK phosphorylation.
However, VEGF-induced phosphorylation of ERK was not significantly affected. In line with these results, silencing of Rap1a and Rap1b did not affect endothelial cell proliferation, which is controlled by the ERK pathway.

Finally, in the present work we provide new evidence that RAPL is an important mediator of Rap1-induced angiogenesis. Silencing of RAPL as well as silencing of Rap1a and/or Rap1b blocked the migration and angiogenic sprouting of endothelial cells. Consistent with the present findings, another group reported that overexpression of a RAPL mutant perturbed wound healing26 and that RAPL is involved in the lymphocyte migration61. Overexpression of constitutive active Rap1aV12 partly rescued the angiogenic sprouting defect induced by silencing of RAPL in endothelial cells, suggesting that other effector proteins may also mediate the Rap1aV12-induced angiogenic sprouting independent on RAPL.

In leukocytes, RAPL associates with the β2-integrin, LFA-162. However, LFA-1 is not expressed in endothelial cells but only in hematopoietic cells and, therefore, another mechanism accounts for the effects of RAPL in endothelial cells. Moreover, we could not detect any direct association of RAPL with the α5- or β1-integrin subunit in coimmunoprecipitation experiments (data not shown). In immunofluorescent stainings for β1-integrins, we only observed to a limited extent colocalization of GFP-RAPL with the β1-integrin-subunit or localization of GFP-RAPL in the vicinity of β1-integrin-subunits near the membrane of migrating cells (data not shown), indicating that the majority of RAPL does not bind directly to the β1-integrin at least in the absence of stimulation with growth factors. It is also conceivable, that RAPL may only transiently associate with the β1-integrins or that RAPL affects only indirectly integrin function in endothelial cells through signaling via other intermediate effector molecules. Another possibility is that RAPL mediates migration and 3-dimensional angiogenic sprouting of endothelial cells downstream of Rap1 independent of integrins. In this regard, it was shown that Rap1/RAPL may affect microtubule extension in endothelial cells26. However, silencing of Rap1a and Rap1b in HUVEC
did not affect the extension of microtubule during migration in a scratch wound assay (data not shown) suggesting that the Rap1/RAPL axis at least in our experimental setting affects migration and angiogenic sprouting through a mechanism distinct of microtubule regulation. Another possibility is, that RAPL may affect downstream of Rap1 the migratory capacity and angiogenic sprouting of endothelial cells through an effect on cell polarization or actin polymerization / protrusion, processes necessary for directional cell migration. Indeed, it was shown that RAPL induces downstream of Rap1 the localization of the CXCR4 receptor to the leading front of leukocytes (polarization)\(^27\) raising the possibility that RAPL may also in endothelial cells affect the localization of growth factor receptors during migration and sprouting. Additional studies are mandatory, in order to elucidate the molecular mechanism by which RAPL affects migration and angiogenic sprouting of endothelial cells downstream of Rap1.

Interestingly, RIAM is another effector of Rap1, which can affect integrin function in leukocytes\(^28\). However, we could not detect RIAM protein expression in endothelial cells (data not shown). Additional studies are required to identify new Rap1 effectors, which are involved in mediating Rap1-induced angiogenesis.

Beyond the regulation of integrin affinity and integrin-dependent adhesion to extracellular matrix proteins, which affect the migratory capacity of endothelial cells and their sprouting activity, Rap1 was also shown to regulate the assembly of VE-Cadherin and to regulate vascular permeability\(^29,31-33\). This is an additional pathway, which could affect angiogenesis.

Taken together, Rap1a and Rap1b are key mediators of angiogenesis by affecting β1-integrin-affinity and integrin-dependent adhesion and migration in endothelial cells. Moreover, the angiogenic effects of Rap1 are mediated at least in part through the effector protein RAPL. It is conceivable, that regulation of Rap1 could be a therapeutical target for anti-angiogenic and pro-angiogenic approaches in order to treat patients with pathological angiogenesis or ischemic disorders.
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Authorship

G. Carmona: performed experiments, designed experiments, manuscript correction, S. Göttig: manuscript correction and contributed to the genotyping, A. Orlandi: performed proliferation experiments and manuscript correction, J. Scheele: provided the Rap1a +/- and Rap1a -/- mice, T. Bäuerle: imaging with contrast enhanced sonography, M. Jugold: imaging with contrast enhanced sonography, F. Kiessling: imaging with contrast enhanced sonography, R. Henschler: manuscript correction, provided the Rap1a +/- and Rap1a -/- mice and contributed to the genotyping, A. Zeiher: manuscript correction, S. Dimmeler: designed experiments, manuscript correction, E. Chavakis: designed experiments, manuscript writing, performed experiments.

Conflict of interest disclosure: The authors declare no competing financial interests
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**Figure Legends:**

**Fig 1: Effect of RapGAP1 overexpression on *in vitro* angiogenesis**

**A,** HUVEC were transfected with Rap1GAP1-FLAG or empty vector. After 24 hours, cells were lysed and subjected to Western blot analysis using a Flag-tag specific antibody. An antibody directed against tubulin was used as loading control. **B,** Three-dimensional *in vitro* angiogenic sprouting in a spheroidal culture system with collagen–embedded spheroids of Rap1GAP1-FLAG- versus mock-transfected endothelial cells in the presence or absence (control) of bFGF 50 ng/mL. The mean cumulative length of sprouts per spheroid was assessed after 24 h (*P<0.05 vs. empty vector, **P<0.05 vs. empty vector+bFGF, n=9).** **C,** Statistical analysis and representative micrographs of the tube-forming activity. HUVEC were seeded on Matrigel Basement Membrane Matrix 24 hours after transfection with the indicated plasmids (n=3). The length of capillary-like structures/networks was measured in five different high power fields by light microscopy after 24 hours (*P<0.05 vs. empty vector) Bar, 200µm. **D,** Three-dimensional *in vitro* angiogenesis with collagen–embedded spheroids of Rap1a-, Rap1aV12- or mock-transfected HUVEC (n=7) (*P<0.05 vs. empty vector). **E,** Three-dimensional *in vitro* angiogenic sprouting in a spheroidal culture system with collagen–embedded endothelial spheroids of Rap1aV12- or mock-transfected HUVEC. The sprouting assay was performed in the presence of blocking monoclonal β1-integrin antibodies or murine isotype control antibodies. Data are presented as mean ± SEM (n=5, *P<0.05 vs. Rap1aV12+IgG).

**Fig 2: Silencing of Rap1 inhibits *in vitro* angiogenesis**

Endothelial cells transfected with siRNAs targeted against Rap1a, Rap1b, or scrambled controls. Two different sequences were used as indicated by I and II. **A,** Expression of Rap1a and Rap1b mRNA was assessed by RT-PCR, GAPDH serves as loading control. **B,** Statistical
analysis and representative images of spheroidal sprouting assay performed with endothelial cells transfected as indicated with Rap1a-, Rap1b-, Rap1a- and Rap1b-siRNA (Sequence I) or scrambled siRNA in the absence or presence of bFGF 50 ng/mL (n=13). The mean cumulative length of sprouts per spheroid was assessed after 24 h (*P<0.05 vs. scrambled siRNA, #P<0.05 vs. scrambled siRNA + bFGF). Bar, 100 µm.

**Fig 3: Silencing of Rap1 inhibits endothelial migration**

**A,** HUVEC cells were transfected with GFP-RBD$_{RalaGDS}$. 6 hours after transfection, a wound was created. Representative images of three different experiments, are depicted, showing recruitment of the GFP-RBD$_{RalaGDS}$ (as an indicator of Rap1 activity) at the leading edge of migrating endothelial cells. **B,** HUVECs were transfected with Rap1GAP1-FLAG or empty vector. After 24 hours, cells were seeded in the upper chamber of modified Boyden chambers coated with fibronectin (n=5) or collagen (n=5). Endothelial cell migration was stimulated using VEGF (50 ng/mL) as chemoattractant where indicated. Data are presented as mean migrated cells % of control ± SEM (*P<0.05 vs. empty vector, **P<0.05 vs. empty vector+ VEGF). **C,** Migration assay on fibronectin (n=5) with endothelial cells transfected with siRNAs targeted against Rap1a, Rap1b Rap1a/Rap1b, or scrambled siRNA. After 48 hours, cells were seeded in the upper chamber of modified Boyden chambers. Endothelial cell migration was assessed using VEGF (50 ng/mL) as chemoattractant. Data are presented as mean migrated cells % of control ± SEM (*P<0.05 vs. scrambled siRNA, #P<0.05 vs. scrambled siRNA + VEGF).

**Fig 4: Inhibition of Rap1 reduced endothelial adhesion**

**A,** HUVEC were transfected with Rap1GAP1-FLAG or empty vector and adhesion assays were performed with transfected endothelial cells. After 24 hours, cells were allowed to adhere for one hour on fibronectin (n=6) or collagen (n=9) (*P<0.05 vs. empty vector). **B,** Adhesion assay
with endothelial cells transfected with siRNAs targeted against Rap1a, Rap1b, Rap1a/Rap1b, or scrambled siRNA. After 48 hours, cells were allowed to adhere for one hour on fibronectin-coated wells (n=5). Data are presented as % of adhering cells ± SEM (*P<0.05 vs. scr). C, 12 hours after transfection HUVEC cells were grown on 4-well chamber slides. 48 hours after transfection serum-starved cells were left untreated or stimulated with 8-pCPT-2'-O-Me-cAMP 100 µM for 10 min. Immunofluorescence was performed using HUTS21 antibodies. Representative pictures from 3 different experiments are depicted. Bar, 10 µm.

**Fig 5: Altered angiogenic cell signaling in Rap1-silenced endothelial cells**

Endothelial cells transfected with siRNAs targeted against Rap1a/Rap1b, or scrambled siRNA. A, After 48 hours, HUVEC were serum-starved for 3 hours and then left untreated or stimulated with VEGF (50ng/mL) for 5 min. Cell lysates were subjected to phospho-Akt1-Ser473- or total Akt1-ELISA analysis. B, After 48 hours, HUVEC were serum-starved for 3 hours and then left untreated or stimulated with VEGF (50 ng/mL) for 10 min. Cell lysates were subjected to Western blot analysis using phospho Erk1/2 or total Erk1/2. C, Transfected HUVEC were lysed 48 h after transfection. Cell lysates were subjected to Western blot analysis using antibodies against phospho-Y397-FAK, FAK, Rap1 and tubulin.

**Fig 6: Role of Rap1a for neovascularisation capacity in vivo**

A/B, Rap1a+/+ (n=11), Rap1a+/− (n=11) and Rap1a−/− (n=4) mice were subjected to hind limb ischemia as described in the methods section. A, Capillary density (ratio of the number of capillaries to the number of myocytes) was determined in 8 µm-frozen sections of ischemic muscles. Representative images of ischemic muscles are shown on the left panel (CD31, red fluorescence; laminin, green fluorescence). A quantitative analysis of capillary density is shown. Data are presented as mean ± SEM (*P<0.05 versus Rap1a+/+). Bar, 20µm. B, Conductance
vessels in the adductor muscles were identified by size and smooth muscle actin staining (SMA) using a Cy3-labeled mouse monoclonal antibody for smooth muscle actin. The number of small (<50 µm), medium (50–100 µm), and large vessels was determined separately. Data are presented as mean ± SEM (*P<0.05 versus Rap1a+/+, <50 µm). Evaluation was performed in a blinded fashion. C, The perfusion of ischemic limbs was assessed by high frequency ultrasound in Rap1a−/− and Rap1a+/+ (wild type) mice. D, Statistical summary of blood vessel infiltration in Matrigel sections stained with an anti-smooth muscle actin antibody in wild-type and Rap1a+/− mice. Quantitative results are presented as mean ± SEM; n = 4 (Rap1a+/+), n = 4 (Rap1a−/−). Evaluation was performed in a blinded fashion. Sections of Matrigel plugs were stained with hematoxylin and eosin (H&E), Bar, 20 µm (right panel).

**Fig 7: RAPL contributes to the angiogenic effect of Rap1a**

Endothelial cells transfected with siRNAs targeted against RAPL or scrambled control. A, 24 hours later, expression of RAPL mRNA was assessed by RT-PCR, GAPDH serves as loading control. B, Statistical summary of spheroid assays with RAPL siRNA or scrambled transfected endothelial cells. Spheroids were stimulated with or without bFGF 50 ng/mL (n=3). Cumulative length of all sprouts originating from each spheroid was quantified after 24 hours. Statistical summary represents the mean ± SEM (*P<0.05 vs. scrambled siRNA, #P<0.05 vs. scrambled siRNA + bFGF). C, 48 hours after transfection migration assays on fibronectin were performed (n=5). Data are presented as mean migrated cells % of control ± SEM (*P<0.05 vs. scrambled siRNA, #P<0.05 vs. scrambled siRNA + VEGF). D, RT-PCR analysis of RAPL and Rap1aV12 expression by RT-PCR. GAPDH serves as loading control. E, Statistical summary and representative micrographs of spheroid assay performed to analyze the effect of simultaneous RAPL silencing and Rap1aV12 overexpression on sprouting capacity of HUVEC (n=5). Data are
given as mean ± SEM (*P<0.05 vs. scrambled siRNA + Rap1aV12; **P<0.05 vs. scrambled siRNA +pcDNA3.1; #P<0.05 RAPL siRNA + pcDNA3.1) Bar, 100 µm.
Figure 2: Carmona et al

A

<table>
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<th>Rap1a (280bp)</th>
<th>GAPDH (628bp)</th>
<th>Rap1b (262bp)</th>
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<td>H2O</td>
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B

Sprout formation

Cumulative length per spheroid (μm)

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<tr>
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bFGF
Figure 3: Carmona et al

A. Localization of active GTP-bound Rap1 in migrating endothelial cells

B. Migration

C. Migration on fibronectin
Figure 6: Carmona et al

A

HLI

Caipillary density

Rap1a⁺/⁺ | Rap1a⁺/- | Rap1a⁻/-

CD31-PE

merge

B

HLI

α-SMA⁺ vessels/mm²

<50 μm | 50-100 μm | >100 μm

Rap1a⁺/⁺ | Rap1a⁺/- | Rap1a⁻/-

C

Rap1a⁻/- | Rap1a⁺/+ - high | low

D

Matrigel plug

vessel/microscopic field

Rap1a⁺/+ | Rap1a⁺/-

* indicates statistical significance.
Figure 7: Carmona et al

A

scrambled RAPL siRNA H₂O

RAPL (437bp)

GAPDH (628bp)

B

Sprout formation

Cumulative length per spheroid (µm)

scrambled RAPL siRNA scrambled RAPL siRNA

* #

C

Migration on fibronectin

% of control

scrambled RAPL siRNA scramblde RAPL siRNA

* #

D

Migration on fibronectin

mock V12 mock V12 H₂O

scr scr RAPLsi RAPLsi

Rap1a (280bp)

RAPL (437bp)

GAPDH (628bp)

E

Sprout formation

cumulative length per spheroid (µm)

scrambled + + - - -

RAPL siRNA - - + + +

pCDNA 3.1 his + - + + +

Rap1aV12 his - + - + +

empty vector Rap1aV12

empty vector Rap1aV12
Role of the small GTPase Rap1 for integrin activity regulation in endothelial cells and angiogenesis

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