

# Cyclin-dependent Kinase 5 Regulates Endothelial Cell Migration and Angiogenesis<sup>§</sup>

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Angiogenesis contributes to various pathological conditions. Due to the resistance against existing antiangiogenic therapy, an urgent need exists to understand the molecular basis of vessel growth and to identify new targets for antiangiogenic therapy. Here we show that cyclin-dependent kinase 5 (Cdk5), an important modulator of neuronal processes, regulates endothelial cell migration and angiogenesis, suggesting Cdk5 as a novel target for antiangiogenic therapy. Inhibition or knockdown of Cdk5 reduces endothelial cell motility and blocks angiogenesis *in vitro* and *in vivo*. We elucidate a specific signaling of Cdk5 in the endothelium; in contrast to neuronal cells, the motile defects upon inhibition of Cdk5 are not caused by an impaired function of focal adhesions or microtubules but by the reduced formation of lamellipodia. Inhibition or down-regulation of Cdk5 decreases the activity of the small GTPase Rac1 and results in a disorganized actin cytoskeleton. Constitutive active Rac1 compensates for the inhibiting effects of Cdk5 knockdown on migration, suggesting that Cdk5 exerts its effects in endothelial cell migration via Rac1. Our work elucidates Cdk5 as a pivotal new regulator of endothelial cell migration and angiogenesis. It suggests Cdk5 as a novel, pharmacologically accessible target for antiangiogenic therapy and provides the basis for a new therapeutic application of Cdk5 inhibitors as antiangiogenic agents.

Angiogenesis is involved in various pathological conditions, including arthritis, psoriasis, diabetic retinopathy, macula degeneration, and cancer (1). During recent years, the search for antiangiogenic compounds and their molecular targets has been intensified. Due to its key role in angiogenesis, research initially focused on vascular endothelial growth factor (VEGF). VEGF receptor inhibitors such as the monoclonal antibody bevacizumab (Avastin<sup>®</sup>) as well as VEGF tyrosine kinase inhibitors such as sunitinib (Sutent<sup>®</sup>) or sorafenib (Nexavar<sup>®</sup>) have been approved for cancer therapy. Unfortunately, the benefits of these therapeutics are at best transitory and mostly followed by a restoration of tumor growth and progression (2). This resistance to antiangiogenic therapy causes a great need for new targets to inhibit vessel growth, interfering with steps in the

angiogenic cascade different from the response to a single growth factor.

Cyclin-dependent kinase 5 (Cdk5) is a small serine/threonine kinase belonging to the family of Cdks. In contrast to the cell cycle-related Cdks (*e.g.* Cdks 1, 2, 4, or 6), Cdk5 is not implicated in cell cycle control (3). Instead, it is an important regulator of neuronal development, and it controls various processes in postmitotic neurons (4). Although it is expressed ubiquitously, so far, just a few reports indicate a function of Cdk5 beyond the nervous system. Scarcely anything is known about a potential function of Cdk5 in the vasculature, and its exact functions and signaling mechanisms in the endothelium remain unknown (5–8).

Our aim was to close this gap of knowledge. This is the first study that describes the function of Cdk5 in the endothelium. It focuses on endothelial cell migration and angiogenesis and provides the first information concerning the signaling mechanism of endothelial Cdk5.

## EXPERIMENTAL PROCEDURES

### Cell Culture

HUVECs<sup>2</sup> were prepared by digestion of umbilical veins with collagenase A as described previously and cultured in endothelial cell growth medium (ECGM, Provitro, Berlin, Germany) (9). Umbilical cords were collected from local hospitals in accordance with the declaration of Helsinki. Roscovitine was from Sigma-Aldrich.

### Migration Assay

Confluent HUVECs were scratched with a pipette tip and treated as indicated. After 16 h, cells were fixed with 3% formaldehyde, and images were taken using the TILLvisION system (Lochham, Germany) connected to an Axiovert 200 microscope (Zeiss, Germany). Evaluation of pictures was made by S.CO LifeScience (Garching, Germany). Migration was quantified as the ratio of the area covered with cells and the area of the cell-free wound. Experiments with the proliferation inhibitor 5-hydroxyurea were performed to exclude an influence of anti-proliferative effects in the scratch assay in our setting.

<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental videos 1–5.

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<sup>2</sup> The abbreviations used are: HUVEC, human umbilical vein endothelial cell; ECGM, endothelial cell growth medium; nt, non-targeting; FAK, focal adhesion kinase; ROCK, Rho-associated protein kinase; 5-HU, 5-hydroxyurea; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; rosc, roscovitine.

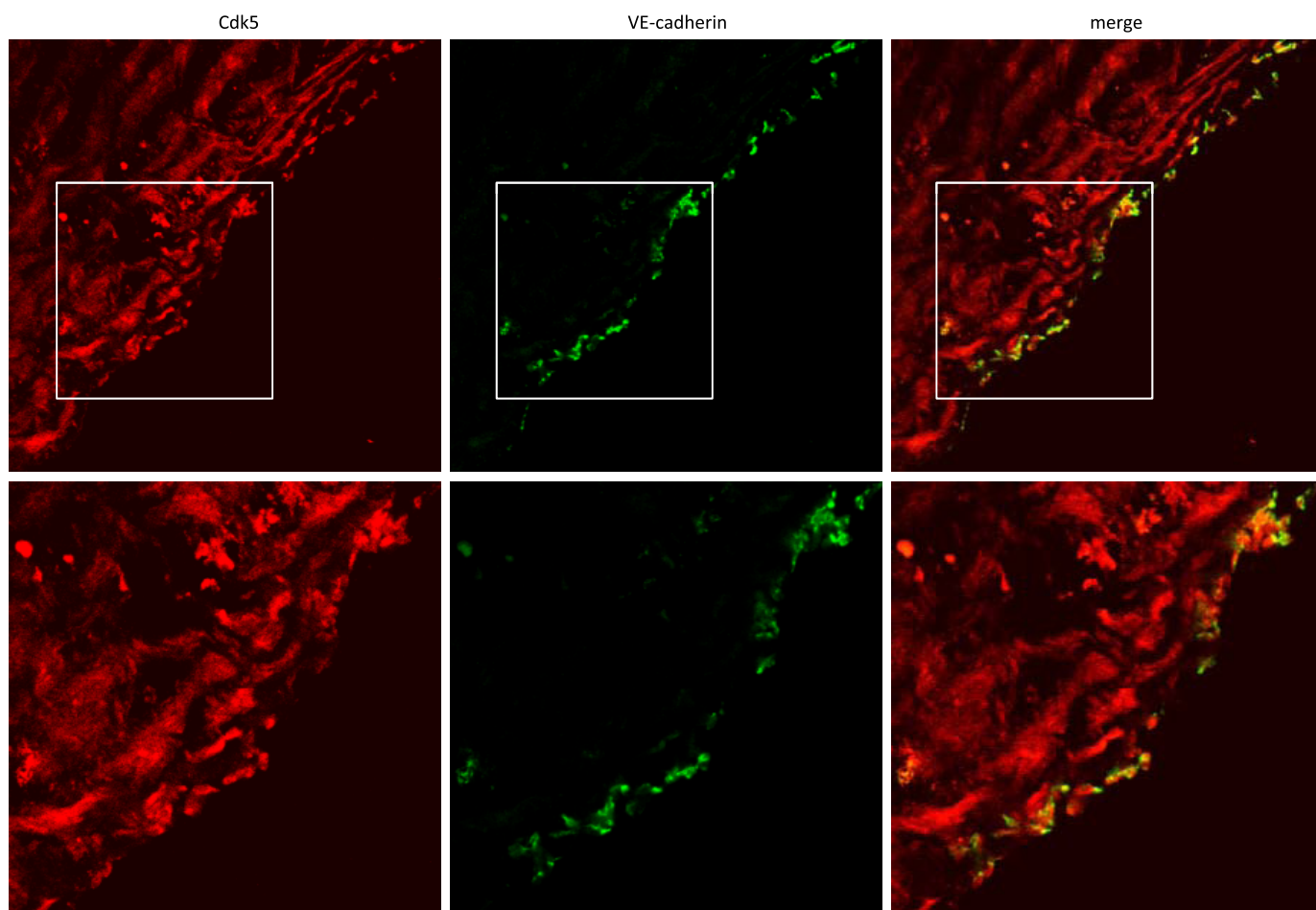


FIGURE 1. **Cdk5 is expressed in the endothelium *in vivo*.** Images display the staining of Cdk5 (red) and VE-cadherin (green) in a human umbilical cord. The lower panels show the area marked by the squares in the upper panels in higher magnification. The merged images show the overlay of the two channels (yellow), demonstrating the localization of Cdk5 to the endothelium.

### Chemotaxis Assay

Cells were seeded into  $\mu$ -Slide chemotaxis (ibidi GmbH, Munich, Germany). After 4 h, an FCS gradient from 0% FCS to 10% FCS was generated, according to the manufacturer's protocol. Images were obtained with a Zeiss LSM 510 META confocal microscope and the appropriate LSM software. The objective used was a Ph1-NEOFLUAR 10 $\times$ /0.30. A heating stage from EMBLEM (Heidelberg, Germany) was used to keep cells at 37  $^{\circ}$ C and 5% CO<sub>2</sub>. Images of cells have been obtained for 20 h.

### Tube Formation Assay

1  $\times$  10<sup>4</sup> HUVECs in ECGM containing roscovitine were seeded onto Matrigel<sup>®</sup> (Schubert & Weiss-OMNILAB, Munich, Germany)-coated ibidi angiogenesis-slides (ibidi GmbH). After 16 h, images were taken using the TILLvisION system. Evaluation of pictures was performed by S.CO Life-Science. Tube length (displayed in red in Fig. 2) was analyzed.

### Chorioallantoic Membrane Assay

Fertilized white leghorn chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) were incubated at 37  $^{\circ}$ C for 72 h with constant humidity. Eggs were transferred into dishes (*ex ovo*),

incubated for further 72 h, and stimulated with VEGF (1 ng/disk) alone or with VEGF and roscovitine (45  $\mu$ g/disk) using small cellulose disks. The next day, chorioallantoic membranes were photographed using a stereomicroscope (Olympus, Munich, Germany).

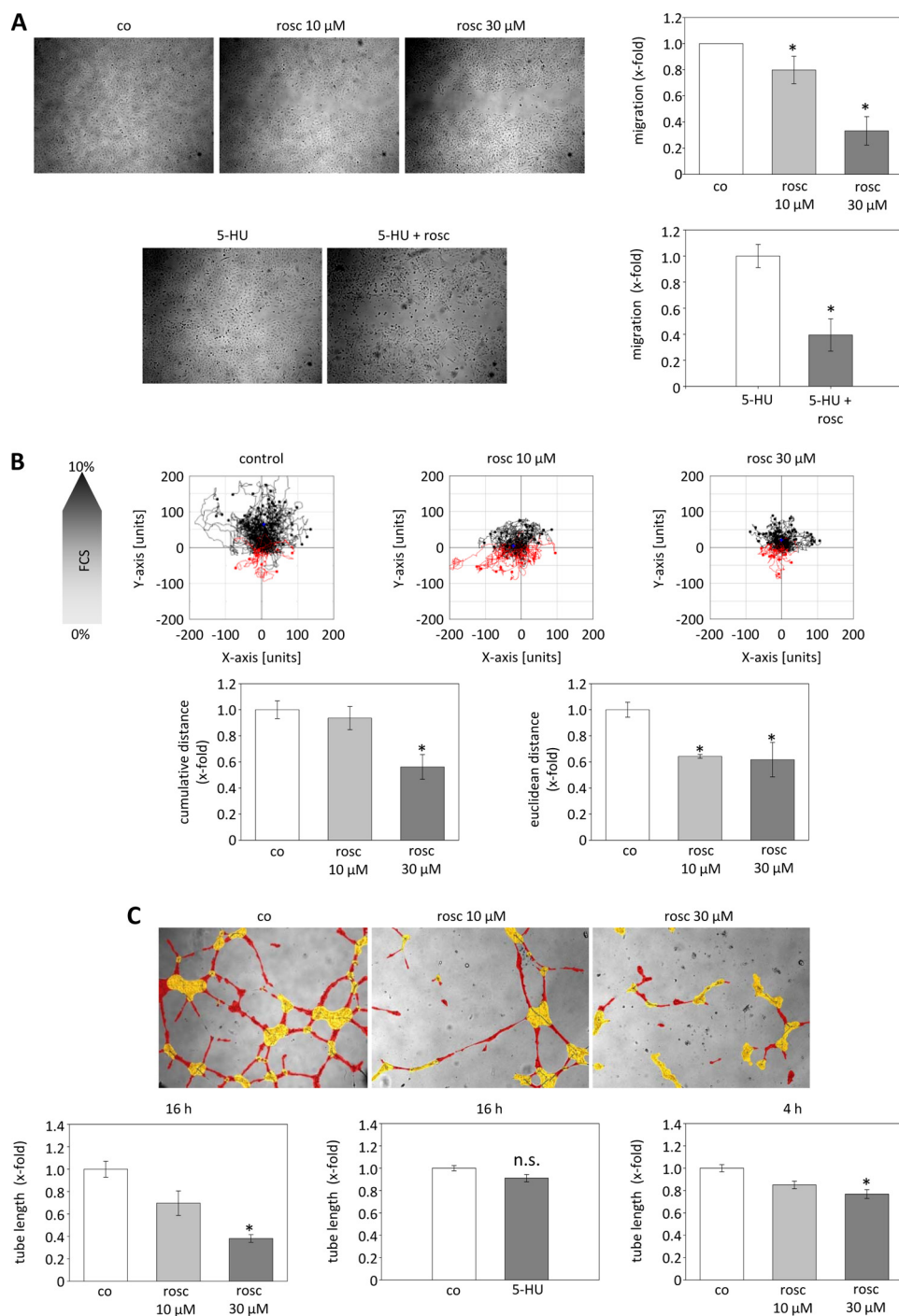
### Mouse Aortic Ring Assay

Mouse aortic rings were embedded into Matrigel. Once endothelial cell sprouting occurred, rings were treated with either ECGM or ECGM containing roscovitine. After 72 h, images were taken using the TILLvisION system.

### Cornea Micropocket Assay

Both eyes of C57BL/6J 8-week-old female mice were implanted with pellets containing basic FGF (80 ng/pellet) as described previously (10, 11). Starting at the time of surgery, mice (20–21 g of body weight) were injected daily with either DMSO (control) or roscovitine for 5 days. For each application, 200  $\mu$ l of the solutions were injected intraperitoneally, and mice received 100 mg/kg of body weight of roscovitine each time. On postoperative day 6, vascularization was quantified. Vessel length (VL) and clock hours (CH) were measured, and the vascularized area (VA) was calculated as  $1/2\pi \times VL \times 0.4 CH$ . The study complies with the

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**FIGURE 2. Inhibition of Cdk5 reduces angiogenesis in vitro.** *A*, inhibition of Cdk5 by roscovitine (rosc) decreases endothelial cell migration (Kruskal-Wallis one-way ANOVA on ranks, \*,  $p < 0.05$ ,  $n = 4$ ; upper panels). Treatment with 5-HU (2 mM) alone had no effect on HUVEC migration and did not alter the migration-inhibiting effect of roscovitine (Student's *t* test, \*,  $p < 0.001$ ;  $n = 5$ ; lower panel). *B*, inhibition of Cdk5 disturbs chemotaxis of HUVECs. Untreated cells (co) move along the FCS gradient (0–10%, upper left panel). rosc at 10  $\mu$ M inhibits cell orientation (upper middle panel) and reduces cell motility at 30  $\mu$ M (upper right panel), respectively. Cells migrating in the direction of the FCS are shown in black, and cells migrating in other directions are in red (one-way ANOVA/Dunnett's test, \*,  $p < 0.05$ ,  $n = 3$ ). *C*, inhibition of Cdk5 reduces endothelial tube formation. The images in the upper panel show tube formation of cells with or without rosc (16 h). Tube structures identified by the software are displayed in red, and quantitative evaluations are displayed in the lower panels. rosc (16 h) reduces tube length, and treatment with 5-HU (2 mM, 16 h) had no effect. Tube formation is reduced upon inhibition of Cdk5 after 4 h, excluding the effects of roscovitine on cell proliferation (roscovitine, 16 h, one-way ANOVA/Dunnett's test, \*,  $p < 0.05$ ,  $n = 3$ ; 5-HU, 16 h, Kruskal-Wallis one-way ANOVA on ranks, \*,  $p < 0.05$ ,  $n = 4$ ; roscovitine, 4 h, one-way ANOVA/Dunnett's test, \*,  $p < 0.05$ ,  $n = 4$ ). n.s., not significant.

Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (47) and with local regulations at the University of Debrecen.

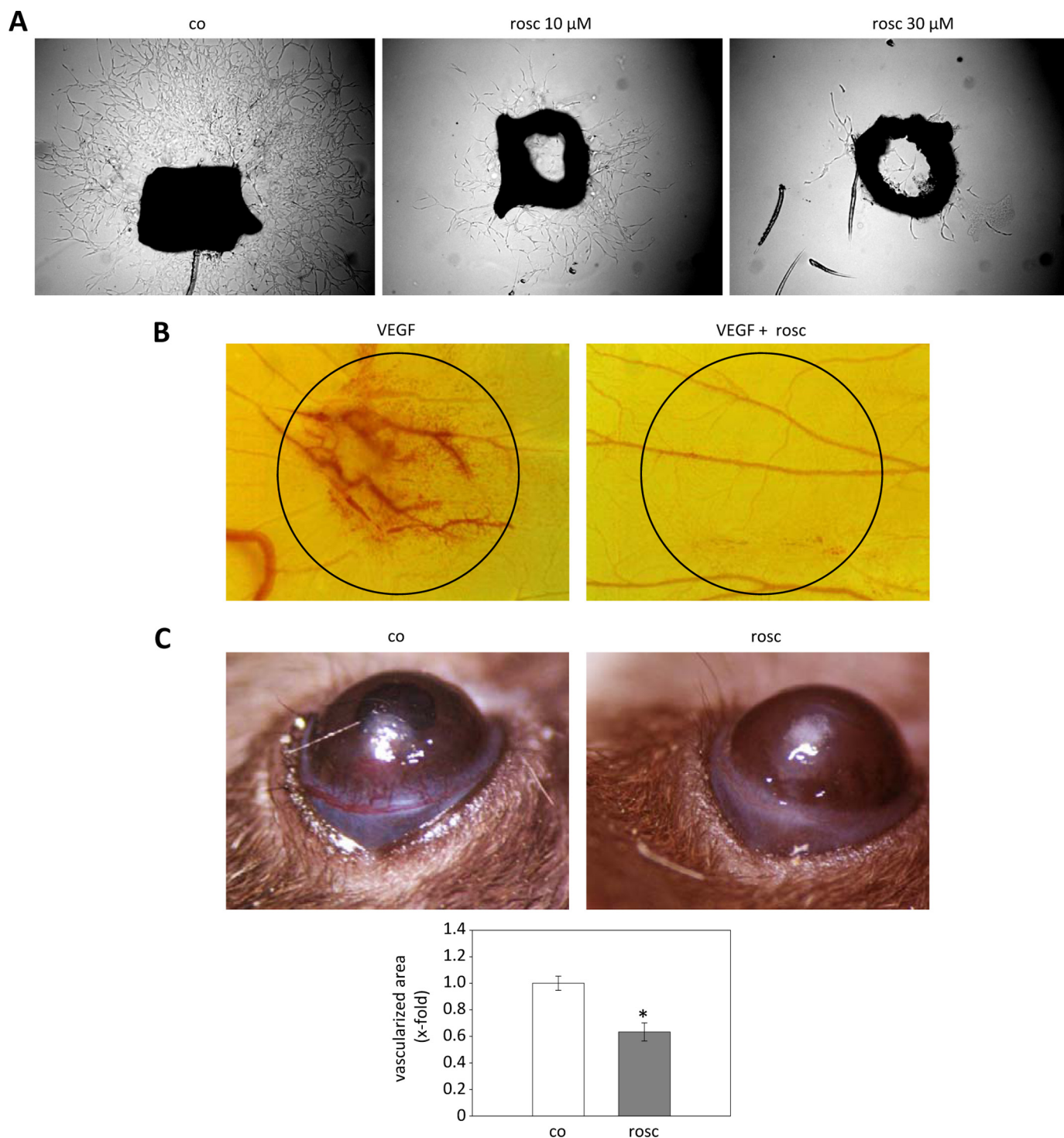
### Transfection

**Cdk2 and Cdk5 siRNA**— $2 \times 10^6$  HUVECs were transfected with 3.0  $\mu$ g of ON-TARGETplus Cdk2 siRNA (1.5  $\mu$ g of J-003236-1, 5'-PUAUUAGGAUGGUUAAGCU-CUU-3', and 1.5  $\mu$ g of J-003236-12, 5'-PUCUCCCGUCAACUUG-UUUCUU-3'; Dharmacon, Lafayette, CO) or 3.0  $\mu$ g of ON-TARGETplus Cdk5 siRNA (1.5  $\mu$ g of J-003239-09, 5'-PACAUCGG-AUAGGGCUUAUUAU-3', and 1.5  $\mu$ g of J-003239-10, 5'-PGAUCUCAUGAGUCUCCCGGUU-3'), respectively, by electroporation with the Nucleofector<sup>TM</sup>II (Amaxa, Cologne, Germany) according to the manufacturer's protocol. For transfection control, HUVECs were transfected with 3.0  $\mu$ g of ON-TARGETplus non-targeting siRNA (nt siRNA) (D-001810-01, 5'-UGGUUUACAUGUCG-ACUAA-3'). Silencing of Cdk2 and Cdk5 was examined by Western blot analysis.

**Cdk5 shRNA**—Adenoviral transduction of HUVECs with Cdk5 shRNA and nt shRNA was performed by SIRION BIOTECH GmbH (Munich, Germany). Knockdown of Cdk5 was examined by Western blot analysis.

**Rac V12**—Co-shRNA-treated HUVECs or Cdk5 shRNA-treated HUVECs were transfected with 2  $\mu$ g of Rac V12 (A. Görlach, Munich, Germany) or pcDNA3 (Molecular Probes/Invitrogen, Karlsruhe, Germany). 12 h after transfection, scratch assays were performed. Transfection efficiency was examined by Western blot analysis.

**Cdk5 Overexpression**—HUVECs were transfected with 3  $\mu$ g of wild-type Cdk5 (Cdk5-wt) or a dominant-negative Cdk5 mutant Cdk5-D145N (Cdk5-dn, Addgene, Cambridge, MA; numbers 1871 and 1873, S. van den Heuvel).



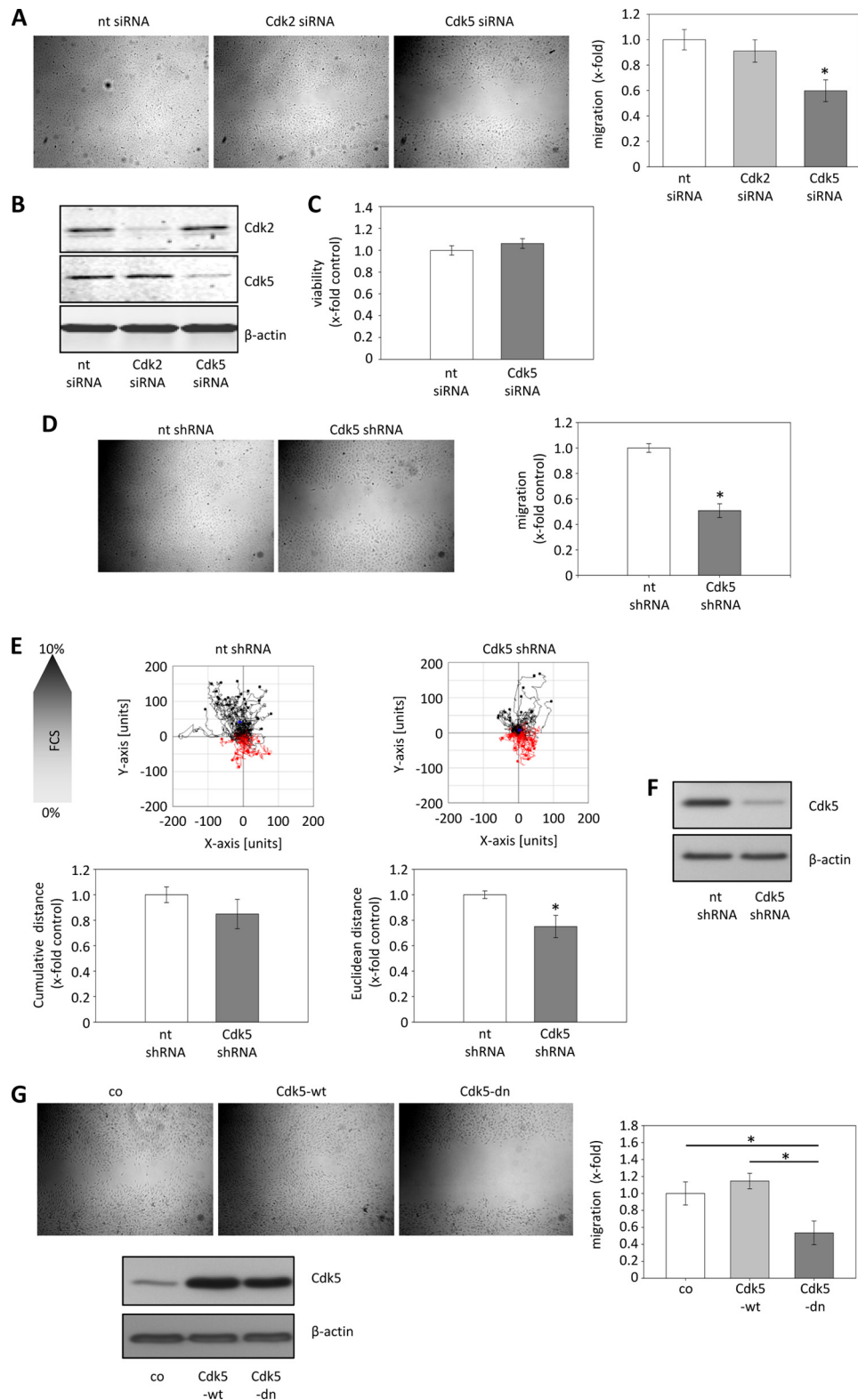
**FIGURE 3. Inhibition of Cdk5 reduces angiogenesis *ex vivo* and *in vivo*.** *A*, inhibition of Cdk5 with rosc inhibits endothelial cell sprouting from mouse aortic rings. *co* indicates mice treated with solvent (DMSO). *B*, inhibition of Cdk5 abolishes VEGF-induced vessel formation in the chorioallantoic membrane assay. *Circles* represent localization of cellulose disks containing VEGF (1 ng/disk) or VEGF combined with rosc (45  $\mu$ g/disk) ( $n = 3$ ). *C*, inhibition of Cdk5 reduces basic FGF-induced neovascularization in the mouse cornea micropocket assay. The growth of blood vessels into the pellet containing basic FGF in mice treated with solvent (DMSO, *co*) is shown in the *upper left panel*. The *upper right panel* indicates one eye of a mouse injected intraperitoneally with rosc (100 mg/kg/day). The *graph (lower panel)* represents the quantitative evaluation of the vascularized area (Student's *t* test corrected for unequal variances,  $p = 0.00018$ ,  $n = 10$  for control,  $n = 16$  for roscovitin).

pCMV-neo-Bam (3  $\mu$ g, Addgene, number 16440, B. Vogelstein) was used as a control. 24 h after transfection, scratch assays were performed. Transfection efficiency was examined by Western blot analysis.

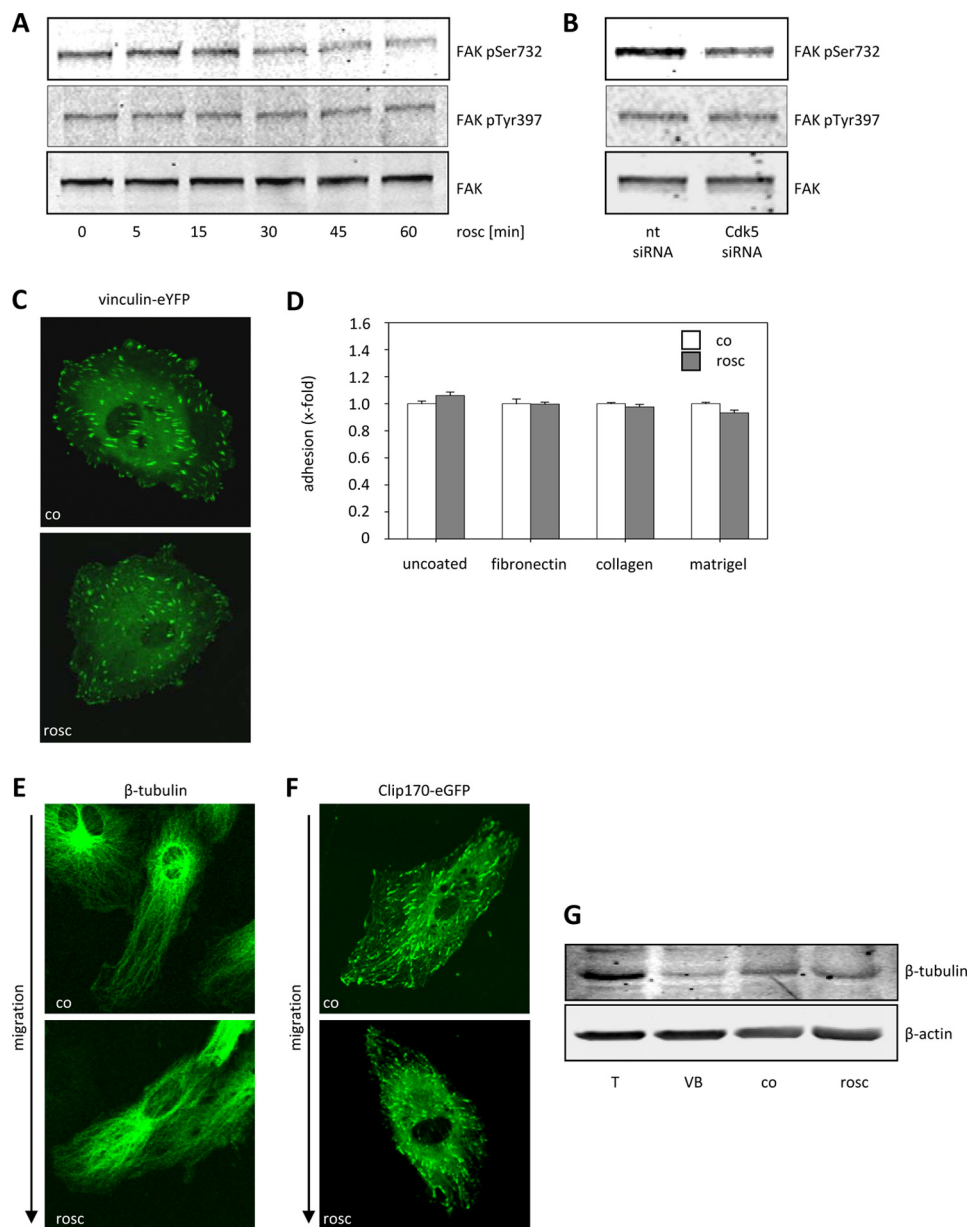
#### CellTiter-Blue Cell Viability Assay

Transfected cells were seeded into 96-well plates ( $2 \times 10^4$  cells/well) for 24 h. CellTiter-Blue<sup>®</sup> assay was performed according to the manufacturer's protocol (Promega Corp.,

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**FIGURE 4. Cdk5 is implicated in the regulation of endothelial cell migration.** *A*, silencing of Cdk5 with siRNA (Cdk5 siRNA) reduces endothelial cell migration; Cdk2 siRNA has no influence, and nt siRNA serves as control (one-way ANOVA/Dunnett's test,  $p < 0.05$ ,  $n = 5$ ). *B*, Cdk2 and Cdk5 are successfully down-regulated 24 h after treatment with siRNA; nt siRNA serves as control, and  $\beta$ -actin indicates equal loading. *C*, Cdk5 siRNA does not influence cell viability (Student's *t* test,  $p = 0.974$ ,  $n = 4$ ). *D*, endothelial cell migration is reduced upon down-regulation of Cdk5 with shRNA; nt shRNA serves as control (Student's *t* test,  $p < 0.001$ ,  $n = 5$ ). *E*, knockdown of Cdk5 inhibits endothelial cell chemokinesis. Cells treated with nt shRNA move along the FCS gradient (0–10%, *left panel*). Cdk5 shRNA inhibits cell orientation (*right panel*). Cells migrating in the direction of FCS are shown in black, and cells migrating in other directions are in red. The quantitative evaluation of cumulative and euclidean distances is displayed in the *lower panels* (Student's *t* test,  $p < 0.05$ ,  $n = 4$ ). *F*, the Western blot indicates successful knockdown of Cdk5 by shRNA; nt siRNA serves as control, and  $\beta$ -actin indicates equal loading. *G*, kinase activity of Cdk5 is required for endothelial cell migration. Scratch assays of HUVECs overexpressing wild-type Cdk5 (Cdk5-wt) or dominant-negative Cdk5 (Cdk5-dn) are shown. Cells transfected with pCMV-neo-Bam (empty vector (co)) were used as control. Dominant-negative Cdk5 significantly decreases HUVEC migration (one-way ANOVA/Holm-Sidak,  $p < 0.05$ ,  $n = 4$ ).



**FIGURE 5. Cdk5 does not influence cell adhesion and microtubules.** *A*, roscovitin decreases the phosphorylation of FAK at Ser-732; the phosphorylation of FAK at Tyr-397 is not influenced. *B*, Cdk5 siRNA reduces the phosphorylation of FAK specifically at Ser-732. *C*, Cdk5 inhibition does not influence the size, localization, and dynamics of focal adhesions. *co* indicates the absence of rosc. *D*, rosc (30  $\mu$ M) does not influence the adhesion of cells on differentially coated surfaces (Student's *t* test, *n* = 3). *E*, microtubule structure is not changed by Cdk5 inhibition. *F*, Cdk5 inhibition does not influence the dynamics of microtubules. Representative images show HUVECs expressing eGFP-CLIP-170 during migration in the absence (*co*) or presence of rosc (30  $\mu$ M) (*n* = 2). *G*, rosc (30  $\mu$ M) does not influence the polymerization of tubulin. Taxol (*T*) and vinblastine (*VB*) show the expected stabilization or fragmentation of microtubules, respectively. Fractions of polymerized tubulin are shown (*n* = 3).

Madison, WI). Fluorescence was measured at 560 nm using a TECAN SPECTRAFluor Plus fluorescence, absorbance, and luminescence reader (MTX Lab Systems, Inc., Crailsheim, Germany).

#### Western Blot Analysis

Western blot analysis was performed as described previously (12). Antibodies against Cdk5 were from Molecular Probes/Invitrogen, antibodies against focal adhesion kinase (FAK) and FAK phospho-Tyr-397 were from Santa Cruz Biotechnology

(Santa Cruz, CA), antibodies against p27<sup>kip1</sup> and FAK phospho-Ser-732 were from BIOSOURCE (Camarillo, CA), and the antibody against Myc tag was from Cell Signaling (Danvers, MA). Alexa Fluor 680 goat-anti-mouse and Alexa Fluor 800 goat-anti-rabbit were used as secondary antibodies (Molecular Probes/Invitrogen). For detection, an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used.

#### Adhesion Assay

HUVECs were trypsinized and suspended in ECGM or ECGM containing roscovitin (30  $\mu$ M), and  $1 \times 10^6$  cells/well were plated in 24-well plates, which were either left uncoated or coated with fibronectin (25  $\mu$ g/ml), collagen (0.001% in PBS) or Matrigel (10% in serum-free medium) After 30 min, cells were stained with crystal violet, and absorption was measured using the TECAN SPECTRAFluor Plus fluorescence, absorbance, and luminescence reader.

#### Confocal Laser Scanning Microscopy

Primary antibodies against Rac1 (Millipore, Billerica, MA) and cortactin (Cell Signaling Technology, Denver, MA) were diluted 1:100 in PBS containing 0.2% BSA. F-actin was stained with rhodamine/phalloidin (1:400, R 415, Molecular Probes/Invitrogen).

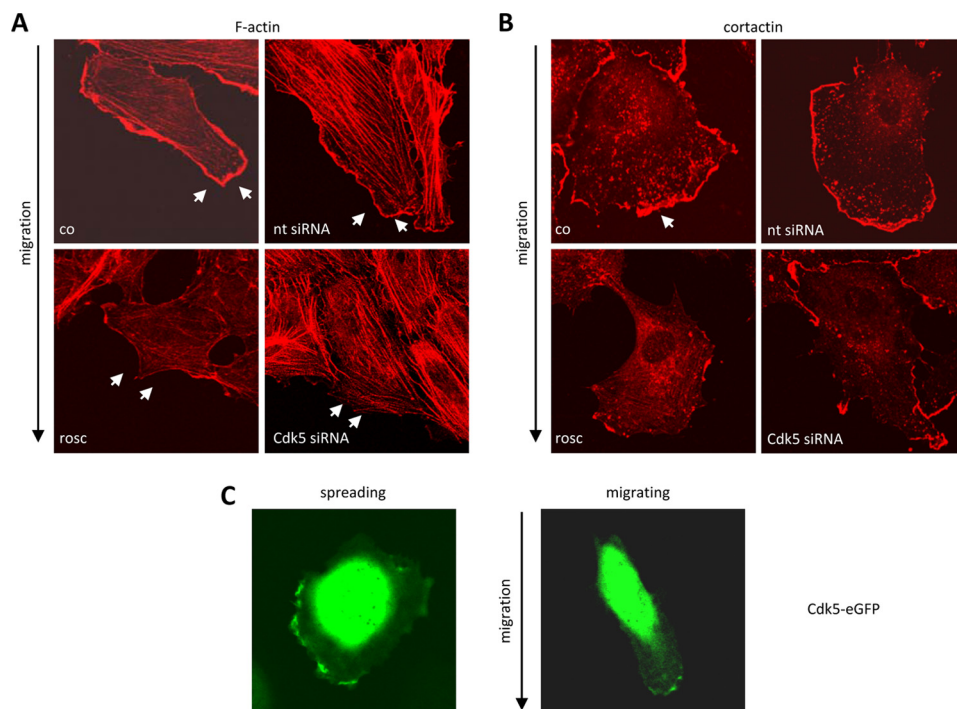
For live cell imaging, cells were transfected with the indicated plasmids, and experiments were started 24 h (eGFP-Cdk5) or 48 h (YFP-Rac1, eGFP-CLIP-170, eYFP-vinculin) after transfection, respectively. eGFP-CLIP-170 was kindly provided by N. Galjart (Rotterdam, The

Netherlands), eYFP-vinculin was provided by A. Bershadsky (Rehovot, Israel), eYFP-Rac1 was from ATCC/Promochem (Wesel, Germany), and eGFP-Cdk5 was from Addgene (Cambridge, MA; number 1346, L.-H. Tsai). Images were obtained with a Zeiss LSM 510 META confocal microscope and the appropriate LSM software.

#### Tubulin Fractionation

Cells were treated as indicated for 4 h and lysed using lysis buffer containing Pipes (100 mM), glycerol (2 M), Triton-X-100

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**FIGURE 6. Cdk5 regulates the formation of lamellipodia.** *A* and *B*, inhibition of Cdk5 using rosc (30  $\mu\text{M}$ ) or Cdk5 siRNA reduces the formation of lamellipodia during migration. *co* indicates untreated cells. *A*, migrating HUVECs stained for F-actin ( $n = 3$ ). *B*, migrating cells stained with anti-cortactin antibodies ( $n = 3$ ). *C*, Cdk5 localizes to lamellipodia. Images represent HUVECs overexpressing Cdk5-eGFP during spreading (*left panel*) and migration (*right panel*), respectively (each  $n = 3$ ).

(0.5%),  $\text{MgCl}_2$  (2 mM), EGTA (2 mM), taxol (5  $\mu\text{M}$ ), GTP (1 mM), phenylmethylsulfonyl fluoride (PMSF) (1 mM), and Complete<sup>®</sup> protease inhibitor (Roche Applied Science) (4%). Lysates were centrifuged (45 min, 47,000 rpm), and the supernatant was mixed with 3 $\times$  Laemmli sample buffer and boiled for 5 min. The pellet was incubated with 40  $\mu\text{l}$  of a buffer containing Tris/HCl (100 mM),  $\text{MgCl}_2$  (1 mM), and  $\text{CaCl}_2$  (10 mM) at 4  $^\circ\text{C}$  for 60 min, mixed with 3 $\times$  Laemmli sample buffer, and boiled for 5 min. Western blot analysis was performed using anti- $\beta$ -tubulin antibody (Santa Cruz Biotechnology).

### Pulldown Assay

Cells were treated as indicated and plated for 30 min. Pull-down assays were performed according to the manufacturer's protocol (Rho activation assay kit 17-294 and Rac1 activation assay kit 17-441, both from Millipore, Billerica, MA).

### Statistical Analysis

The number of independently performed experiments and the statistical tests used are stated in the respective figure legends. Graph data represent means  $\pm$  S.E. Statistical analysis was performed with the SigmaStat software Version 3.1 (SYSTAT Software, Inc., Point Richmond, CA). Statistical significance is assumed if  $p \leq 0.05$ .

## RESULTS

**Cdk5 Is Expressed in the Endothelium**—To demonstrate the presence of Cdk5 in the endothelium *in situ*, human umbilical cords were stained for Cdk5 (red) together with VE-cadherin (green), which was used as endothelial marker (Fig. 1). As

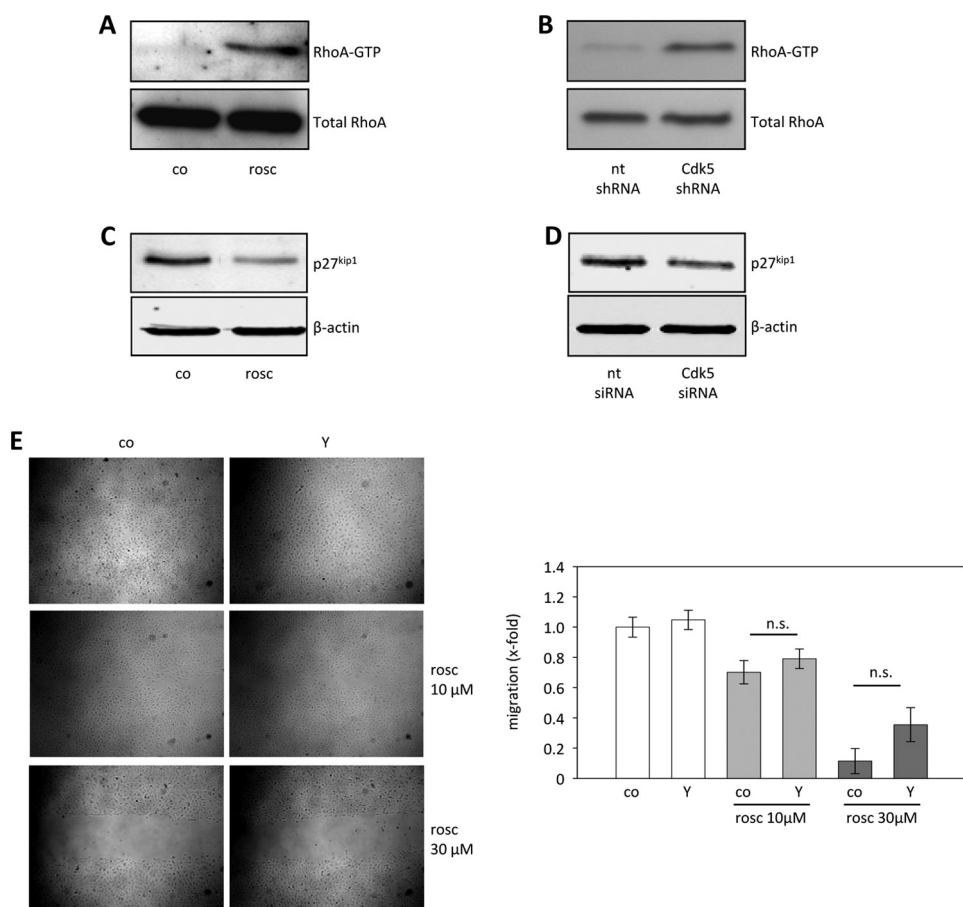
expected, Cdk5, which is expressed ubiquitously (4), localizes to all tissues in the human umbilical cords. Merged images clearly show the expression of Cdk5 in the endothelium (overlay of Cdk5 and VE-cadherin, yellow).

**Inhibition of Cdk5 with Roscovitine Disrupts Angiogenesis**—The impact of inhibition of Cdk5 on angiogenesis was examined by performing various functional angiogenesis assays *in vitro* and *in vivo* using the Cdk5 inhibitor roscovitine. Inhibition of Cdk5 by roscovitine significantly reduced endothelial cell migration by 20% (10  $\mu\text{M}$ ) and 67% (30  $\mu\text{M}$ ), respectively (Fig. 2*A*, upper panel). Possible false positive results due to antiproliferative effects have been excluded by using 5-hydroxyurea (5-HU), an inhibitor of proliferation. Treatment with 5-HU alone did not influence wound closure, and the migration-inhibiting effect of roscovitine was not altered in the presence of 5-HU (Fig. 2*A*, lower panel). Chemotaxis

assays revealed that untreated cells moved along an FCS (0–10%) gradient (Fig. 2*B*, upper left panel). Cells treated with 10  $\mu\text{M}$  roscovitine still moved (indicated by an intact cumulative distance) but did not follow the FCS gradient, suggesting a loss of orientation (indicated by a reduced euclidean distance). Roscovitine at a concentration of 30  $\mu\text{M}$  reduced both cumulative and euclidean distance, reflecting a completely defective motility (Fig. 2*B*, lower panels). Applying tube formation assays, untreated cells formed three-dimensional structures. In contrast, tube formation of roscovitine-treated cells was reduced significantly after 16 h. Again, the antiangiogenic effects of roscovitine are not caused by an inhibition of proliferation. The treatment with 5-HU (16 h) had no effect on tube formation, and roscovitine significantly reduced tube length already after 4 h treatment (Fig. 2*C*). Supplemental video 1 shows cells seeded on Matrigel for 16 h. Cells organize themselves into tube-like structures by changing their shape and establishing contacts to neighboring cells, but they do not proliferate significantly during tube formation.

Inhibition of Cdk5 impaired endothelial cell sprouting out of aortic rings (Fig. 3*A*). By applying chorioallantoic membrane assays, we found a strong induction of vessel formation by VEGF, which was completely blocked upon inhibition of Cdk5 (Fig. 3*B*). The mouse cornea micropocket assay demonstrated a pronounced reduction of basic FGF-induced neovascularization *in vivo* (Fig. 3*C*) upon intraperitoneal administration of roscovitine (100 mg/kg/day).

**Cdk5 Is Required for Endothelial Cell Migration**—To verify the function of Cdk5 in angiogenesis, Cdk5 was specifically down-regulated using RNAi. Two different approaches were



**FIGURE 7. Cdk5 influences the p27<sup>kip1</sup>/RhoA pathway.** *A*, inhibition of Cdk5 increases the amount of active GTP-bound RhoA (RhoA-GTP). Total RhoA serves as loading control ( $n = 3$ ). *co* indicates untreated cells. *B*, knockdown of Cdk5 with shRNA (*Cdk5 shRNA*) increases the amount of RhoA-GTP in comparison with cells treated with nt shRNA. Total RhoA serves as loading control ( $n = 3$ ). *C*, inhibition of Cdk5 decrease p27<sup>kip1</sup> protein expression. Cells were either left untreated or treated with rosc (30  $\mu\text{M}$ ,  $n = 3$ ). *D*, p27<sup>kip1</sup> expression of HUVECs transfected with Cdk5 siRNA or with nt siRNA, respectively, is shown ( $n = 3$ ). *E*, preincubation with Y27632 (Y, 10  $\mu\text{M}$ ) does not significantly abolish the effect of rosc (10 and 30  $\mu\text{M}$ ) on HUVEC migration. Representative images show migrating cells treated as indicated. The bar graph shows the quantitative evaluation (one-way ANOVA/Holm-Sidak,  $n = 3$ ). *n.s.*, not significant.

used: silencing of Cdk5 with siRNA (nucleofection) and down-regulation of Cdk5 using shRNA (adenoviral transfer). In scratch assays, Cdk5 siRNA reduced the migration of HUVECs by 40%. In contrast, the silencing of Cdk2, another prominent target of roscovitine, showed no significant effect (Fig. 4A) (13). Successful down-regulation of the proteins was determined 24 h after transfection, and no cross-reaction was observed (Fig. 4B). The viability of cells transfected with Cdk5 siRNA was not changed (CellTiter-Blue assay, Fig. 4C). Down-regulation of Cdk5 by shRNA resulted in a very similar effect; by applying scratch assays, cells treated with Cdk5 shRNA showed a reduced migration by 49% (Fig. 4D). Cdk5 shRNA also significantly reduced the euclidean distance of HUVECs in a chemotactic gradient (Fig. 4E), suggesting a defective chemokinesis (loss of orientation). Fig. 4F shows successful knockdown of Cdk5 with shRNA.

To find out whether Cdk5 kinase activity is required for endothelial cell migration, we examined migration of HUVECs overexpressing wild-type Cdk5 (Cdk5-wt) or a dominant-negative mutant Cdk5-D145N (Cdk5-dn) in comparison with HUVECs overexpressing the empty vector (pCMV-neo-Bam)

serving as control. Overexpression of dominant-negative Cdk5 (kinase-dead Cdk5-D145N) reduced migration by 46%, an extent similar to RNAi experiments. The Western blot shows overexpression of the respective Cdk5 mutants (Fig. 4G).

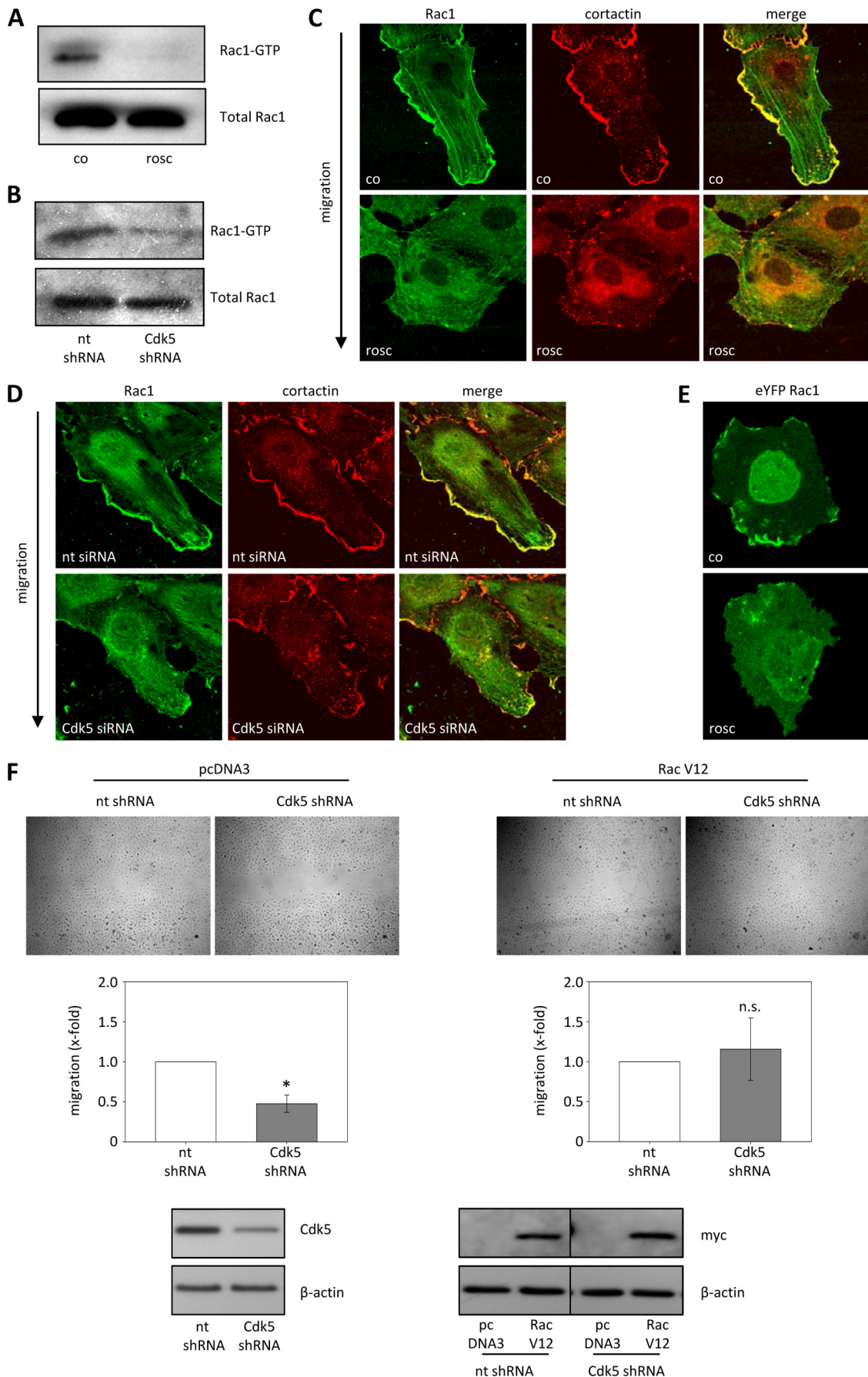
**Cdk5 Does Not Influence Cell Adhesion and Microtubules—**Inhibition and silencing of Cdk5 reduced the phosphorylation of FAK at Ser-732. The autophosphorylation site of FAK at tyrosine 397 was not affected (Fig. 5, A and B). The functional impact of inhibition of Cdk5 on focal adhesion dynamics and adhesion as well as microtubule organization of HUVECs has been analyzed. Focal adhesions were visualized by overexpressing eYFP-vinculin. Inhibition of Cdk5 did not change focal adhesion structure (maturation) and dynamics during cell spreading (Fig. 5C). Furthermore, cell adhesion on various substrates was not affected (Fig. 5D). Moreover, no effect of Cdk5 inhibition on the structure of the tubulin cytoskeleton in migrating HUVECs was found (Fig. 5E). To analyze microtubule dynamics, we overexpressed eGFP-CLIP-170. Treatment with roscovitine neither changed microtubule dynamics (Fig. 5F) nor had any influence on the polymerization of tubulin in contrast to classical tubulin-tar-

geting compounds such as taxol, which stabilizes microtubules, or vinblastine, which causes fragmentation of microtubules (Fig. 5G).

**Cdk5 Influences the Actin Cytoskeleton—**The influence of Cdk5 on the actin cytoskeleton in endothelial cells was examined by analyzing F-actin distribution in migrating cells. Untreated cells and cells treated with nt siRNA formed lamellipodia with a densely packed actin seam at the leading edge (Fig. 6A, upper panels), in contrast to cells treated with roscovitine or Cdk5 siRNA (Fig. 6A, lower panels). In addition, the inhibition of Cdk5 reduced the localization of cortactin, a lamellipodial marker, to the leading edge of migrating cells (Fig. 6B) (14). Overexpression of Cdk5-eGFP in HUVECs demonstrated the localization of Cdk5 to lamellipodia during cell spreading (Fig. 6C, left panel; supplemental video 2) and cell migration (Fig. 6C, right panel; supplemental video 3), suggesting a function of Cdk5 in the formation of lamellipodia.

**Cdk5 Regulates the Activity of RhoA and Rac1—**In pulldown assays, inhibition or down-regulation of Cdk5 increased levels of GTP-bound active RhoA (Fig. 7, A and B), accompanied by a decrease of p27<sup>kip1</sup> protein expression (Fig. 7, C and D). In

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scratch assays, preincubation with the Rho-associated protein kinase (ROCK) inhibitor Y27632 (Y) could not significantly compensate for the inhibition of migration upon treatment with roscovitine (Fig. 7E).

The activity of Rac1 was dramatically decreased upon inhibition and knockdown of Cdk5 (Fig. 8, A and B). Furthermore, inhibition or down-regulation of Cdk5 abolished the localization of Rac1 and its effector cortactin to the leading edge of migrating cells (Fig. 8, C and D). In live cell imaging experiments with cells overexpressing Rac1-eYFP, untreated cells showed a regular cell shape with Rac1 localized to the cell periphery (Fig. 8E, upper panel; supplemental video 4). Inhibition of Cdk5 caused a distribution of Rac1 over the whole cell, accompanied by an irregular cell shape and an irregular formation of ruffles (Fig. 8E, lower panel; supplemental video 5). In scratch assays, overexpression of a constitutively active Rac mutant (Rac V12) compensated for the inhibition of HUVEC migration upon down-regulation of Cdk5 by shRNA (Fig. 8F).

## DISCUSSION

In the present study, we demonstrate a crucial role of Cdk5 in the regulation of endothelial cell migration and angiogenesis. We identify the Cdk5 inhibitor roscovitine as antiangiogenic compound and propose Cdk5 as the target of roscovitine responsible for its antiangiogenic effects. We provide information concerning the signaling of endothelial Cdk5 suggesting that Cdk5 regulates endothelial cell migration via the small GTPase Rac1.

Roscovitine (Seliciclib, CYC202) is a well established Cdk inhibitor, classically developed to control cell proliferation. It has anticancer activity and is currently being evaluated in a phase 2b clinical trial concerning cancer therapy ("Efficacy Study of Oral Seliciclib to Treat Non-Small Cell Lung Cancer").<sup>3</sup> We show that roscovitine functionally blocks angiogenesis *in vitro* and *in vivo*. Roscovitine does not selectively inhibit one specific Cdk (13). It was used as a tool to get first impressions about a potential function of Cdk5 in angiogenesis.

We propose that Cdk5 is the target of roscovitine responsible for its antiangiogenic effects. By selectively down-regulating Cdk5 using RNAi and by overexpressing a kinase-dead Cdk5 mutant, we identify Cdk5 activity as key for the effects on EC migration. Cdk5 classically is known to regulate neuronal processes, and only recently, some reports investigated the role of Cdk5 in cancer cells (15–19). This is the first study that characterizes the function of Cdk5 in the context of angiogenesis.

Our results suggest a new therapeutic indication of roscovitine and Cdk5 inhibitors in general as antiangiogenic agents. A

probable antiangiogenic application of Cdk5 inhibitors might represent a therapeutic benefit to broaden the spectrum and to overcome the drawbacks of already existing inhibitors of angiogenesis in oncology and other pathological processes involving excessive angiogenesis. Increased invasiveness of cancer cells in response to antiangiogenic therapy has just recently been identified as a resistance mechanism to escape nutrient and oxygen deprivation (20–22). In this respect, roscovitine could be of dual benefit as it inhibits both metastasis and angiogenesis (19, 23). Moreover, the inhibition of Cdk5 blocked angiogenesis independently of the pro-angiogenic stimulus used. Thus, the regulation of Cdk5 in the endothelium via one specific growth factor is rather unlikely. This turns Cdk5 into a highly attractive target for antiangiogenic therapy as endothelial cells are able to adapt to antiangiogenic treatment with VEGF inhibitors by up-regulating alternative signaling circuits (2).

Our mechanistic studies implicate that Cdk5 regulates endothelial cell migration via the actin cytoskeleton. We investigated the effects of Cdk5 on cell adhesion, the microtubules, and the actin cytoskeleton, which represent the three central elements regulated during migration (24).

The inhibition of Cdk5 influenced neither cell adhesion nor focal adhesions. This might be surprising as the phosphorylation of FAK at Ser-732 is decreased upon inhibition or down-regulation of Cdk5. A possible explanation might be that FAK kinase activity is not dependent on the phosphorylation at Ser-732 (25). Tyr-397, the initial FAK autophosphorylation site, is not changed upon inhibition of Cdk5. Moreover, the role of the phosphorylation of FAK at Ser-732 in EC migration is not completely clear. In endothelial cells, Ser-732 of FAK was reported to regulate centrosome function during mitosis. The authors suggest that Ser-732 phosphorylation of FAK is crucial for FAK regulation of proliferation and tubulogenesis but not of migration of endothelial cells (26). In contrast, FAK has also been shown to be phosphorylated at Ser-732 by ROCK in ECs, which triggers the formation of ventral focal adhesions and plays a role in VEGF-induced EC migration (27). In our system, besides the decreased phosphorylation of FAK at Ser-732, we found an increase of active RhoA, the most prominent activator of ROCK, upon inhibition/down-regulation of Cdk5. If one considers that ROCK induces the phosphorylation of FAK at Ser-732, one would expect an increased phosphorylation of FAK at Ser-732 upon increased RhoA. This discrepancy might be a result of the complex interplay between FAK and Rho-GTPases. Another study shows that Cdk5 regulates epithelial cell adhesion, cytoskeletal contraction, and migration via modulating RhoA activity by suppressing Src and p190RhoGAP. In contrast to our findings, this work shows a decrease of active RhoA

<sup>3</sup> Belani, C. (2006) *Efficacy Study of Oral Seliciclib to Treat Non-small Cell Lung Cancer* (ClinicalTrials.gov, identifier NCT00372073).

**FIGURE 8. Cdk5 influences Rac1.** A, inhibition of Cdk5 with rosc (30  $\mu$ M) reduces the amount of active GTP-bound Rac1. Total Rac1 serves as loading control ( $n = 3$ ). *co* indicates untreated cells. B, knockdown of Cdk5 with shRNA (Cdk5 shRNA) decreases the amount of Rac1-GTP in comparison with cells treated with nt shRNA. Total Rac1 serves as loading control ( $n = 3$ ). C and D, rosc (30  $\mu$ M) as well as Cdk5 siRNA inhibit localization of Rac1 (green) and cortactin (red) to lamellipodia of migrating endothelial cells. Untreated cells (*co*) or nt siRNA-treated cells serve as the respective controls ( $n = 3$ ). E, during cell spreading, inhibition of Cdk5 abrogates the localization of Rac1 to the cell periphery. Representative images display cells overexpressing Rac1-eYFP during spreading untreated or treated with rosc (30  $\mu$ M), respectively ( $n = 3$ ). F, overexpression of constitutively active Rac1 (Rac V12) compensates for the migration-inhibiting effect of Cdk5 knockdown. Upper panels, in scratch assays, knockdown of Cdk5 with shRNA significantly reduces migration of cells treated with the empty vector (pcDNA3). In HUVECs overexpressing constitutive active Rac1 (Rac V12), treatment with Cdk5 shRNA does not reduce migration. The bar graphs display the quantitative evaluation (pcDNA3, rank sum test, \*,  $p < 0.05$ ,  $n = 5$ ; Rac V12, rank sum test;  $n = 5$ ). Lower panels, the Western blots show the knockdown of Cdk5 by shRNA and overexpression of Myc-tagged Rac V12. *n.s.*, not significant.

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concomitant to an increased migration upon inhibition of Cdk5 (28). Talin is another target of Cdk5 that regulates focal adhesions and migration of neuroblastoma cells. Cdk5-dependent phosphorylation of the talin head domain at Ser-425 prevents its ubiquitylation and degradation, controlling adhesion stability and cell migration (23).

Nonetheless, because we found no change of focal adhesions or cell adhesion upon inhibition of Cdk5, we searched for a different target of Cdk5 in endothelial cell migration. We also did not find any effect of Cdk5 on microtubules, although Ser-732 phosphorylation on FAK previously has been shown to influence microtubule organization, nuclear movement, and neuronal migration (25). Thus, we propose a specific signaling of Cdk5 in the vasculature.

We identify the actin cytoskeleton to be the relevant target of Cdk5 in endothelial cell migration. Endothelial Cdk5 regulates the formation of lamellipodia, actin-based structures that are essential for cell migration (29). Our findings are in line with reports concerning the function of Cdk5 in neurons. Neuronal Cdk5 affects the actin cytoskeleton by phosphorylating p27<sup>kip1</sup> and PAK1 as well as Neurabin-I (25, 30, 31); it controls dendritic spine morphology via phosphorylation of the RhoA guanine-nucleotide exchange factor (GEF) ephexin1 (32); and it phosphorylates the actin-binding proteins WAVE1 and WAVE2 (33, 34).

The small Rho GTPases RhoA and Rac1 represent the most prominent regulators of the actin cytoskeleton (35, 36). We found that inhibition of Cdk5 increased RhoA activity, concomitant to a decrease of p27<sup>kip1</sup> protein level. This parallels findings in neurons, where Cdk5 has been shown to phosphorylate and stabilize p27<sup>kip1</sup>, suppressing RhoA activity and increasing activated cofilin, which regulates actin turnover (31). The migration defect of p27<sup>kip1</sup>-null fibroblasts is rescued by the inhibition of ROCK, the most prominent downstream effector of RhoA (38). In endothelial cells, in contrast, blockade of ROCK could not compensate for the antimigratory effect of roscovitine. Thus, although Cdk5 indeed modulates RhoA activity, it seems not to regulate endothelial cell migration primarily via the p27<sup>kip1</sup>/RhoA/ROCK pathway. In the context of epithelial cell migration, Cdk5 was described as a regulator of RhoA activity and cytoskeletal contraction. In contrast to our findings, in epithelial cells, inhibition of Cdk5 blocks RhoA-ROCK signaling and increases migration (28). The discrepancies concerning the different effects of Cdk5 on the activity of RhoA and the distinct functional consequences might be due to the diverse cell types and suggest a specific signaling of Cdk5 in the endothelium.

Our data indicate Rac1 to be the most likely link between Cdk5 function and endothelial cell migration. We show that inhibition or down-regulation of Cdk5 dramatically reduces Rac1 activity and impairs the localization of Rac1 and its effector cortactin, a regulator of the protrusion and integrity of lamellipodia, to the cell membrane (39). Constitutively active Rac1 compensates for the migration-inhibiting effect of the knockdown of Cdk5. Thus, we propose that Cdk5 exerts its effects in endothelial cell migration via Rac1. This can be interpreted contrariwise to the report of Nikolic and colleagues (30), where neuronal Cdk5 has been elucidated as a downstream

effector of Rac1. A different and much more interesting probable explanation might be a feedback loop between Cdk5 and Rac1. As a kinase, Cdk5 might regulate Rac1 by the phosphorylation of a guanine nucleotide exchange factor, a GTPase-activating protein (GAP), or a GDP dissociation inhibitor (GDI) for Rac1, respectively. Neuronal Cdk5 was shown to phosphorylate various regulators of Rac1. Cdk5-mediated phosphorylation of RasGRF2 down-regulates the activity of Rac1, and the regulation of Trio and Kalirin by Cdk5 activates Rac1 (40–42). By phosphorylating ezrin, Cdk5 was shown to modulate RhoGDI, inhibiting Rac1 (43). The fact that Cdk5 seems to be able to both increase and decrease the activity of Rac1 and, on the contrary, to be regulated by Rac1 suggests that the function of Cdk5 strongly depends on the cellular system and/or on the functional context, respectively.

Our work clearly elucidates a vascular function of Cdk5. Consequently, one would expect a vascular phenotype of Cdk5 knock-out mice. However, vascular or cardiac defects of these mice are not described. According to the crucial function of Cdk5 in the nervous system, Cdk5 knock-out mice show abnormal corticogenesis and neuronal defects and die perinatally (44). Mice exhibiting vascular phenotypes often die during embryogenesis (45, 46) or show neonatal lethality (37), depending on the severity of the phenotype. To make use of endothelial specific Cdk5 knock-out mice might provide the opportunity to study the function of Cdk5 in the endothelium *in vivo*.

In conclusion, this study for the first time presents a novel and crucial function of Cdk5 in endothelial cell migration, elucidates a specific signaling of endothelial Cdk5, and highlights Cdk5 as a promising target for antiangiogenic therapy.

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