

## Cell culture

The cell lines HEK293, HeLa, MS1, PY41, bEND were grown in DMEM supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and antibiotics (penicillin and streptomycin; Invitrogen, Carlsbad, CA). The cell lines Jurkat, K-562, Raji, THP-1, PU5, A20 were grown in RPMI1640 medium supplemented with 10% heat inactivated FCS, 4 mmol/L glutamine, 100 units/ml streptomycin and 100 units/ml penicillin. The primary cells HUVEC (human umbilical vein endothelial cell), HCAEC (human coronary artery EC), HPAEC (human pulmonary artery EC), HMVEC-L (human lung microvascular EC), and HMVEC-C (human cardiac microvascular EC) were obtained from Lonza (Allendale, NJ) and grown in EBM-2 (EC Basal Medium-2) supplemented with EGM-MV SingleQuots (Lonza). HASMC (human aortic smooth muscle cell) were grown in SmBM Basal Medium supplemented with SmGM-2 SingleQuot (Lonza). Mouse primary EC (L-EC and H-EC) were isolated from mouse lung and heart, as described previously.<sup>1</sup>

## siRNA transfections

siRNA transfection in HUVEC was performed as previously described<sup>2,3</sup> using Lipofectamine 2000 (Invitrogen). The luciferase siRNA and mRhoJ siRNA was produced by Dharmacon (Lafayette, CO, cat # P-002099-01 and L-064532-01). Other siRNAs used in this study are listed in Table S1 (Dharmacon).

## Site-directed mutagenesis constructs

Site-directed mutagenesis construct (Mut1, Mut2, and Mut3) at ERG binding site (−44, −30, and −8) within RhoJ promoter was generated using the strategy of two-step PCR for each construct. The resulting plasmids resulted in mutated ERG binding elements AGGAA to AAAAA, or TTCCT to TTTTT. In brief, for single mutants, the wild type human (h) RhoJ promoter described above was used as template. The primers used for the first run were: 1) Mut1: fragment 1-hRhoJ-nest-F and hRhoJ-Mut1-R; fragment 2-hRhoJ-Mut1-F and hRhoJ-nest-R. 2) Mut2: fragment 1-hRhoJ-nest-F and hRhoJ-Mut2-R; fragment 2-hRhoJ-Mut2-F and hRhoJ-nest-R. 3) Mut3: fragment 1-hRhoJ-nest-F and hRhoJ-Mut3-R; fragment 2-hRhoJ-Mut3-F and hRhoJ-nest-R. Primer sequences are listed in Table S3. To generate the double mutant hRhoJ-Mut1/2, hRhoJ-Mut1 was used as template with the same primers as Mut2. The primers used for the second PCR run were hRhoJ-nest-F and hRhoJ-nest-R for all, followed by ligating into the *KpnI-XhoI* site of pGL3 basic.

## Quantitative real-time PCR (QPCR)

Total RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA). Single stranded cDNA was synthesized from total RNA using the RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). SYBR Green I-based real-time PCR was carried out on an Opticon Monitor. The sequences of the primers used in this study are listed in Table S2. For each run, TBP (TATA-box binding protein) primers were used to normalize the amount of cDNA. For samples from laser capture microdissection (LMD), a first amplification of 15 cycles was performed prior to regular QPCR and 18s ribosomal RNA was used for normalization.

## Transactivation assays

HUVEC were plated in a 12-well plate the day before transfection. RhoJ wild-type or mutant promoter constructs were co-transfected with a mammalian expression plasmid (pCI) encoding corresponding genes or empty vector as indicated and 75 ng Renilla (Promega) into HUVEC using Dharmafect I (Dharmacon). After 24 hours of incubation, the cells were lysed in 200  $\mu$ L of

Cell Culture Lysis Reagents (Promega) and analyzed for Luciferase activity by using Luciferase Assay System (Promega) with AutoLumat LB953 (EG&G Berthold, Oak Ridge, TN).

### **Immunohistochemistry**

Primary antibodies diluted in 5% fetal calf serum in PBS, or Hank's balanced salt solution HBSS (Invitrogen) supplemented with 5% FCS for VE-cadherin detection, were applied to acetone fixed tissues according to standard protocols. Rat anti-mouse CD31 Ab was from BD Pharmingen (Palo Alto, CA). Rabbit polyclonal anti-vWF Ab was from DAKO Cytomation (Glostrup, Denmark). Detection was performed with appropriate HRP-conjugated secondary antibodies (all from DAKO Cytomation). Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) and sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

### **Immunofluorescent staining**

Frozen tissue samples were cut at 5  $\mu$ m and stored at  $-80^{\circ}\text{C}$  for future use. Before immunostaining, frozen sections were washed in PBS and permeabilized with 0.5% Triton-X100 for 3 minutes, after which they were blocked in goat serum for 30 minutes at room temperature. The sections were then labeled with anti-RhoJ (Novus Biologicals, Littleton, CO) and anti-mouse CD31 antibody (BD Pharmingen). The secondary antibody labeling was performed with Alexa 594 anti-mouse, or Alexa 488 anti-rat antibodies (Invitrogen). The staining was visualized under fluorescent microscope (Nikon-3000) and images were taken at 40 $\times$  magnification.

### **Western blot analysis**

After corresponding treatments, cells were washed with ice-cold PBS and lysed with RIPA solution, containing a protease inhibitor cocktail. Equal amounts of total protein extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to 0.45- $\mu$ m nitrocellulose membranes. Membranes were blocked with 5% dry milk and probed with respective primary antibodies, rabbit anti-ERG, anti-Cdc42 (Santa Cruz Biotechnology), anti-RhoJ (Sigma), anti-PAK2, anti-p-PAK2, anti-PAK4, anti-p-PAK4, anti-Src, anti-p-Src, anti-p-B-Raf, anti-C-Raf, anti-p-C-Raf, anti-ERK, anti-p-ERK (Cell Signalling, Danvers, MA), and anti-B-Raf (Epitomics, Burlingame, CA), mouse anti-RhoA, anti-Rac1 (Cytoskeleton), anti-tubulin or anti- $\beta$ -actin (Sigma-Aldrich, St. Louis, MO), followed by incubation with HP-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized using an enhanced chemifluorescence detection system (Denville Scientific, Metuchen, NY) according to the manufacturer's protocol and chemiluminescent sensitive film.

### **Chromatin immunoprecipitation (ChIP) assay**

ChIP analysis was performed as previously described<sup>2,3</sup> using the ChIP Assay Kit (Upstate Biotechnology, Waltham, MA) according to the manufacturer's protocols with minor modification. After the DNA was extracted from the supernatant, QPCR was performed using primers hRhoJ-C-F and hRhoJ-C-R (sequences listed in Table S2).

### **Apoptosis assay**

The cell apoptosis was detected using In Situ Cell Death Detection Kit, Fluorecein (Roche, Mannheim, Germany) following manufacturer's instruction. In Brief, HUVEC were plated and transfected with either control or RhoJ siRNA as described previously. After incubation of the

plate for 48 hrs, cells were trypsinized and washed with PBS. After fixation and permeabilization, cells were incubated in TUNEL reaction mixture and analyzed by FACS.

#### **EC cord formation on 2D matrigel**

HUVEC (5000 cells/cm<sup>2</sup>) were treated with either control or RhoJ siRNA (40 nmol/L). After 24 hours of incubation, ECs were plated on matrigel basement membrane matrix. Images were captured after 4–16 hrs incubation.

#### **Tube formation in matrigel *in vivo***

Matrigel injection was performed as described previously.<sup>4</sup> In brief, matrigel mixture containing 2 μM control or RhoJ siRNA, 80 ng/ml bFGF, 64 U/ml heparin, and 250 μl of Matrigel Basement Membrane Matrix (BD Biosciences) was injected subcutaneously into nude mice at flank. The gel plugs were collected after 7 days. After fixation in paraffin, samples were embedded in paraffin, sectioned and stained for hematoxylin and eosin or CD31.

#### **REFERENCES**

1. Jin E, Liu J, Suehiro J, et al. Differential roles for ETS, CREB, and EGR binding sites in mediating VEGF receptor 1 expression *in vivo*. *Blood*. 2009;114:5557–5566.
2. Koh W, Mahan RD, Davis GE. Cdc42– and Rac1–mediated endothelial lumen formation requires Pak2, Pak4 and Par3, and PKC-dependent signaling. *J Cell Sci*. 2008;121:989–1001.
3. Bayless KJ, Davis GE. The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices. *J Cell Sci*. 2002;115:1123–1136.
4. Birdsey GM, Dryden NH, Amsellem V, et al. Transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood*. 2008;111:3498–3506.

**Table S1. Sequences of the primers used for QPCR**

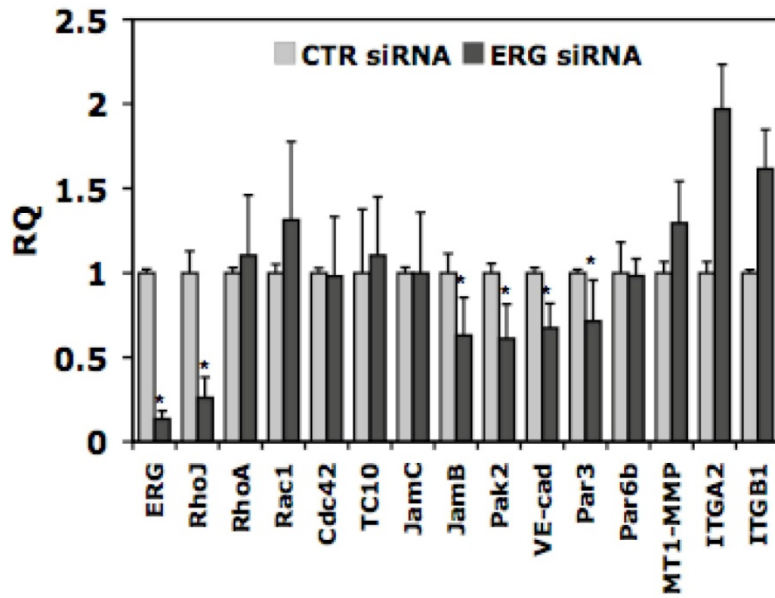
Gene specific siRNA	Sequence of primer (5'-3')
ERG siRNA #1	GAUCCUACGCUAUGGAGUA
ERG siRNA #2	GUGAAUGGCUCAAGGAACU
ERG siRNA #3	GCGCUACGCCUACAAGUUC
ERG siRNA#4	GGACAGACUCCAAGAUGA
RhoJ siRNA #2	UCAUAGGGACCCAGAUUGA
RhoJ siRNA #3	UCAGAAAGGUCUCAAGCG
RhoJ siRNA #4	UGGUUUACAUGUCGACUAA
Control siRNA	UAGCGACUAAACACAUCAA

**Table S2. Sequences of the primers used for QPCR**

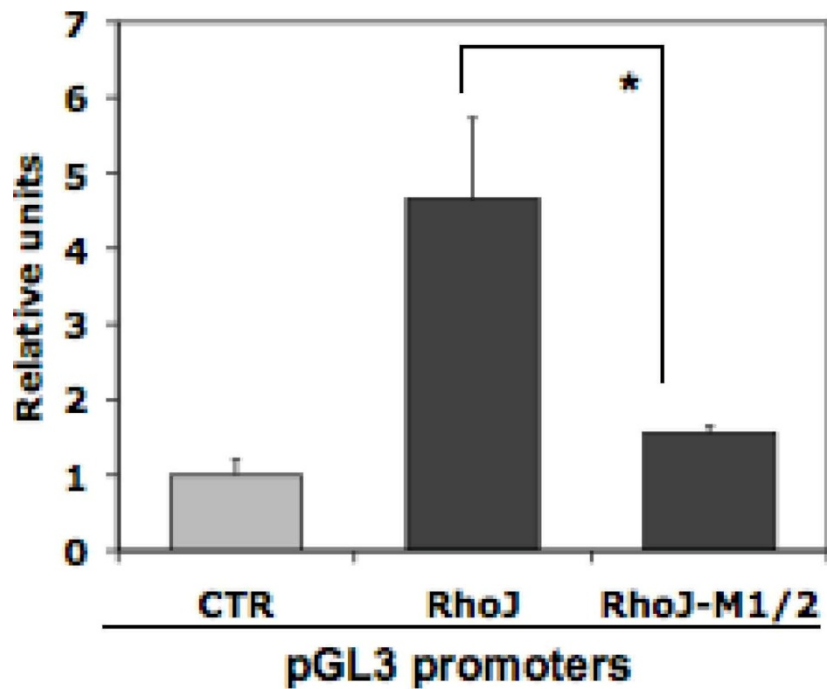
Gene	Sequence of primer (5'-3')
hERG-F	CAGCAGGATTGGCTGTCTCA
hERG-R	CAT TCA CCT GGC TAG GGT TAC AT
hRhoJ-F	GTGCCTTATGTCCTCATAGGGA
hRhoJ-R	GTGACCCTCAGAACAGAGTT
hRhoB-F	CTGCTGATCGTGTTTACAGTAAGG
hRhoB-R	CACCGAGAAGCACATGAGAAT
hRac1-F	GGTGCAGACACTTGCTCTCCTA
hRac1-R	TTCAATGGCAACGCTTCATTC
hRhoG-F	CACAACTAACGCTTTCCCCAA
hRhoG-R	GTGTACGGAGGCGGTCATAC
hTC10-F	CCACCGTCTTCGACCACTAC
hTC10-R	AGGCTGGATTTACCACCGAGA
hCdc42-F	CAGGGCAAGAGGATTATGACAGA
hCdc42-R	TGGAGAGACCACTGAAAAACAGACT
hRhoA-F	TGGAAAGACATGCTTGCTCAT
hRhoA-R	GCCTCAGGCGATCATAATCTTC
hTBP-F	CGTCCCAGCAGGCAACA
hTBP-R	GGTGCAGTTGTGAGAGTCTGTGA
mRhoJ-F	CGGCTGCAATGGACATGAG
mRhoJ-R	GGCACGTATTCCTCTGGGAAG
mTBP-F	ACTTCGTGCAAGAAATGCTGAA
mTBP-R	TGTCCGTGGCTCTTTATTCTCA
hPAR3-F	TCCTTGCCCTGCATCAGCAT
hPAR3-R	GTTACCAAGGCATAGGTGTGCTT
hITGB1-F	CAACGAGGTCATGGTTCATGTT
hITGB1-R	CCAGCTACAATTGGAATGATGTCT
hITGA2-F	ACTCTGTGGCTGCAATTTCTACTG
hITGA2-R	GCACTATCTGGCCGGTATAATTTG
hJamC-F	CTGTGCAAGTGAAGCCAGTGA
hJamC-R	CAGTGTGGCCATCTTGCCTACT
hPAK2-F	GCGACCGGATCATACGAAAT
hPAK2-R	GGCAGCACCATCAACATGTG
hMT1-MMP-F	GAAGCCTGGCTACAGCAATATG
hMT1-MMP-R	TGCAAGCCGTAAAACCTTCTGC
hPARD6B-F	CTGGGCACTATGGAGGTGAAG
hPARD6B-R	ACGTCAACATTGGGGATCTTATG
hVE-cad-F	GAACCCAAGATGTGGCCTTTA G
hVE-cad-R	GATGTGACAACAGCGAGGTGTAA
mVE-cad-F	CAACTTCACCCTCATAAACAACCAT
mVE-cad-R	ACTTGGCATGCTCCCGATT
hRhoJ-C-F	CCTCAGCCCCTGATCCTAA
hRhoJ-C-R	CAGCAGCCTGAGGGTTTTAC
mvWF-F	TGGATCCCGAGTCCTTTGTG
mvWF-R	GAGTACAGGACATGCGCACTCT
mCD31-F	GAGCCCAATCACGTTTCAGTTT
mCD31-R	TCCTTCCTGCTTCTTGCTAGCT

**Table S3. Sequences of the primers used for cloning**

Gene	Sequence of primer (5'-3')
hRhoJ-out-F	ATCGGCCTAGATCCCTGAAT
hRhoJ-out-R	AGAATTCGAGGGGCTTCCTA
hRhoJ-nest-F	CCGGTACCAGGAAAGAAAGGCTTGGACA
hRhoJ-nest-R	AACTCGAGCAGCAGCCTGAGGGTTTTAC
hRhoJ-cDNA-F	TCTGCCGCTTCATGTGCTTTG
hRhoJ-cDNA-R	TGGAGCTTGGCCACAGAATT
hRhoJ-cDNA-nest-F	ATATGAATTCATGAACTGCAAAGAGGGAAC
hRhoJ-cDNA-nest-R	ATATGGATCCTCAGATAATTGAACAGCAGC
hRhoJ-phrGFP-F	ATATGTCGACATGGTGAGCAAGCAGATCCT
hRhoJ-phrGFP-R	ATATGATATCTCAGATAATTGAACAGCAGCT
hRhoJ-Mut1-R	GAAATCATGGGTTTTTTGCTGAATTCCAT
hRhoJ-Mut1-F	ATGGAATTCAGCAAAAACCCATGATTTCC
hRhoJ-Mut2-R	TTGCCGAGTGTCAAAAATCATGGGTTT
hRhoJ-Mut2-F	AAACCCATGATTTTTTGACACTCGGCAA
hRhoJ-Mut3-R	TCCCTTTGCTTTTTCCAGCTTGCCGAGT
hRhoJ-Mut3-F	ACTCGGCAAGCTGGAAAAAGCAAAGGGA



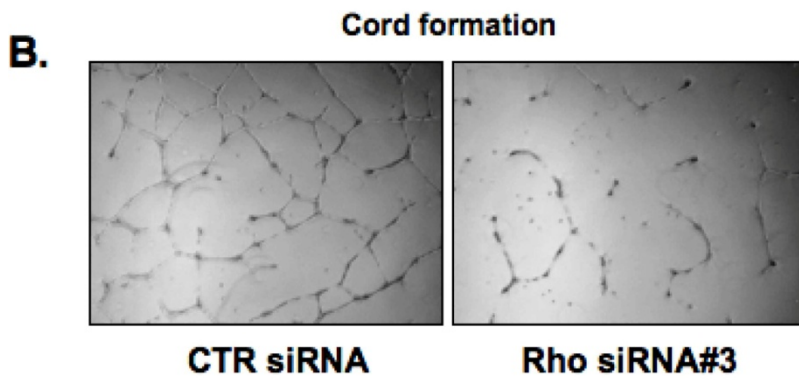
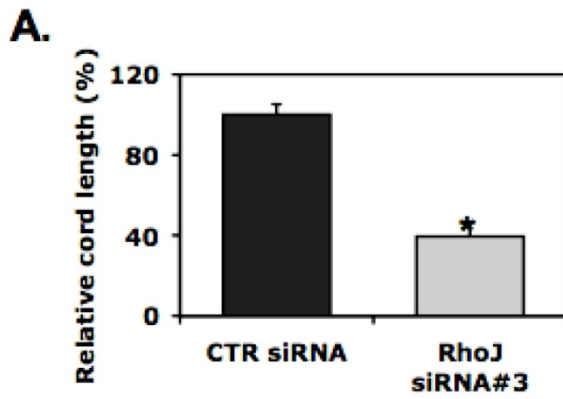
**Figure S1. Identification of downstream targets of ERG that are implicated in EC lumen formation**  
 HUVECs were transfected with indicated siRNA (40 nmol/L). After 48 hours of incubation, RNA was extracted for QPCR. Quantitation of changes in gene expression in ERG siRNA- (dark gray bars) versus control siRNA-treated (light gray bars) HUVEC by QPCR using gene-specific primers. The data are presented relative to control siRNA treated samples.



**Figure S2. Mutation in ERG binding site within RhoJ promoter abrogates the promoter activity in EC**

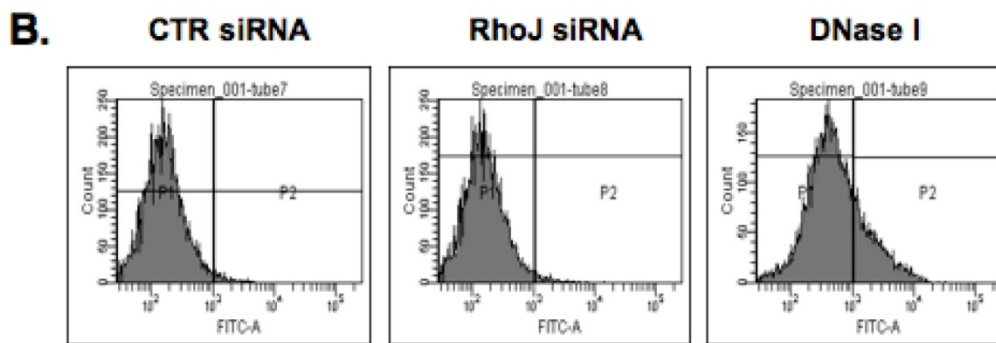
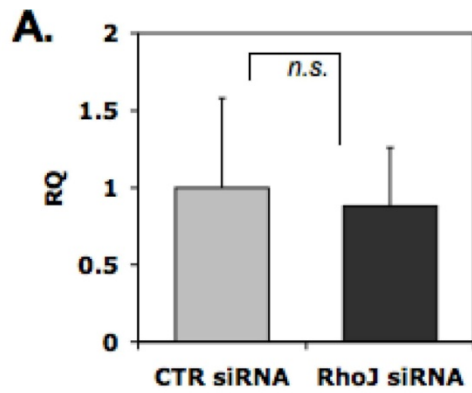
HUVEC were transfected with RhoJ wild-type, double mutant (mut1/2) promoter, or empty pGL3 vector. After 24 hours of incubation, cells were lysed and analyzed for luciferase activity. The data are shown as relative luciferase activity compared with the control plasmid. n=5. \*P<0.05.





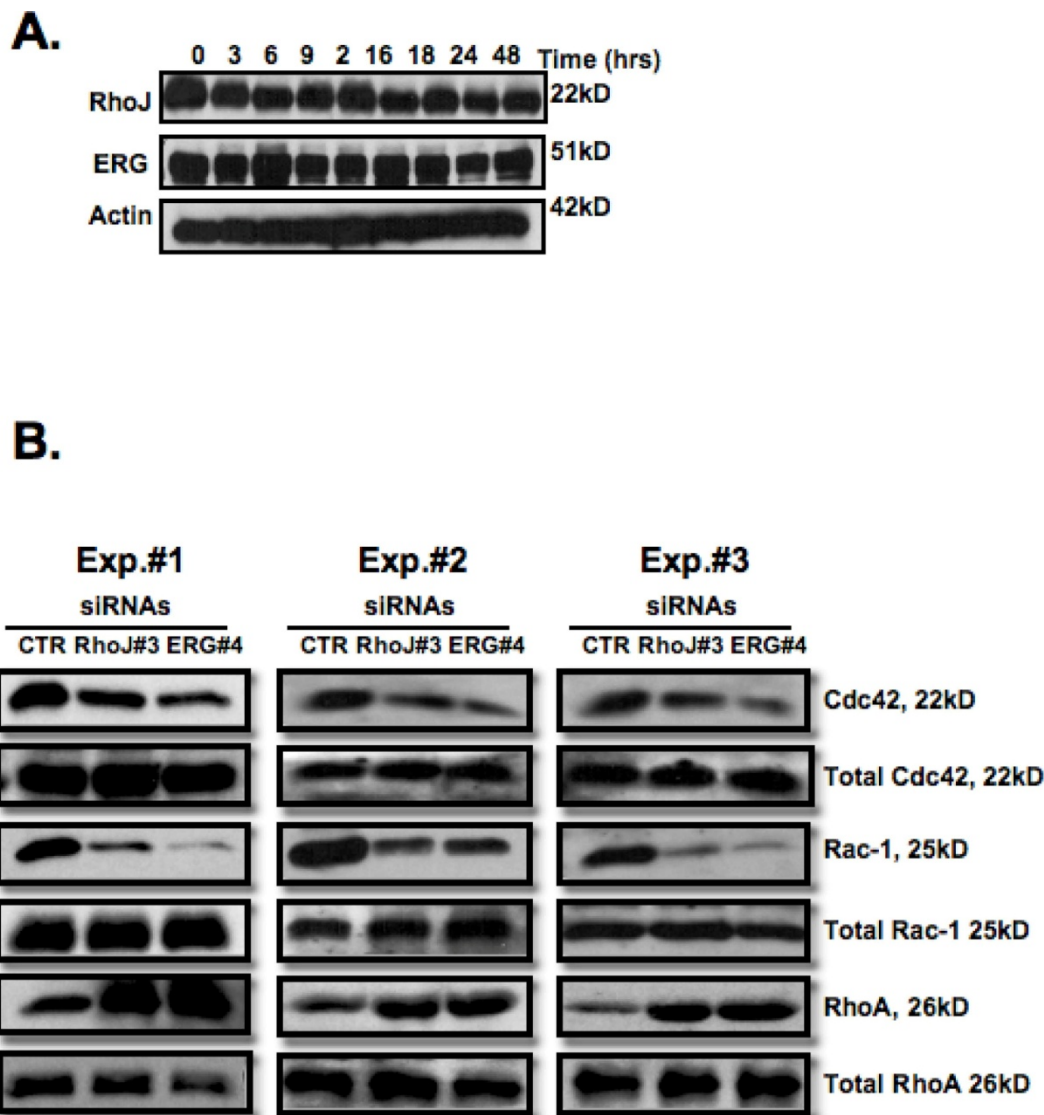
**Figure S3. Effects of RhoJ on EC cord formation on 2D matrigel**

HUVEC were treated with either control or RhoJ siRNA (40 nmol/L). After 24 hours of incubation, ECs were plated on matrigel basement membrane matrix. Images were captured after 4–16 hrs incubation. Cord length was quantified using ImageJ software. n=10. Data are presented as means of relative cord length of each field compared to control siRNA treated samples.



**Figure S4. Effects of RhoJ on EC apoptosis**

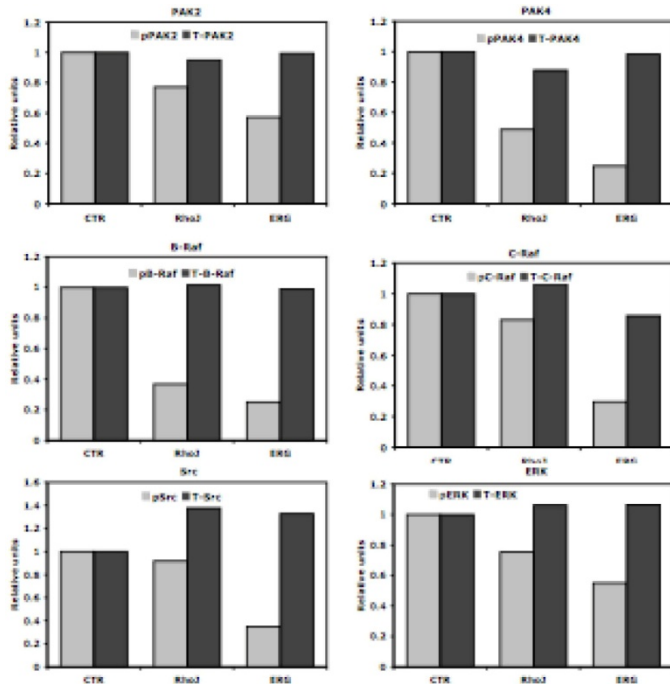
HUVEC were plated and transfected with either control or RhoJ siRNA. After incubation of the plate for 48 hrs, cells were trypsinized and washed with PBS. After fixation and permeabilization, cells were incubated in TUNEL reaction mixture and analyzed by FACS following the manufacturer's instructions. The data are presented relative to control-treated samples. n=4.



**Figure S5**

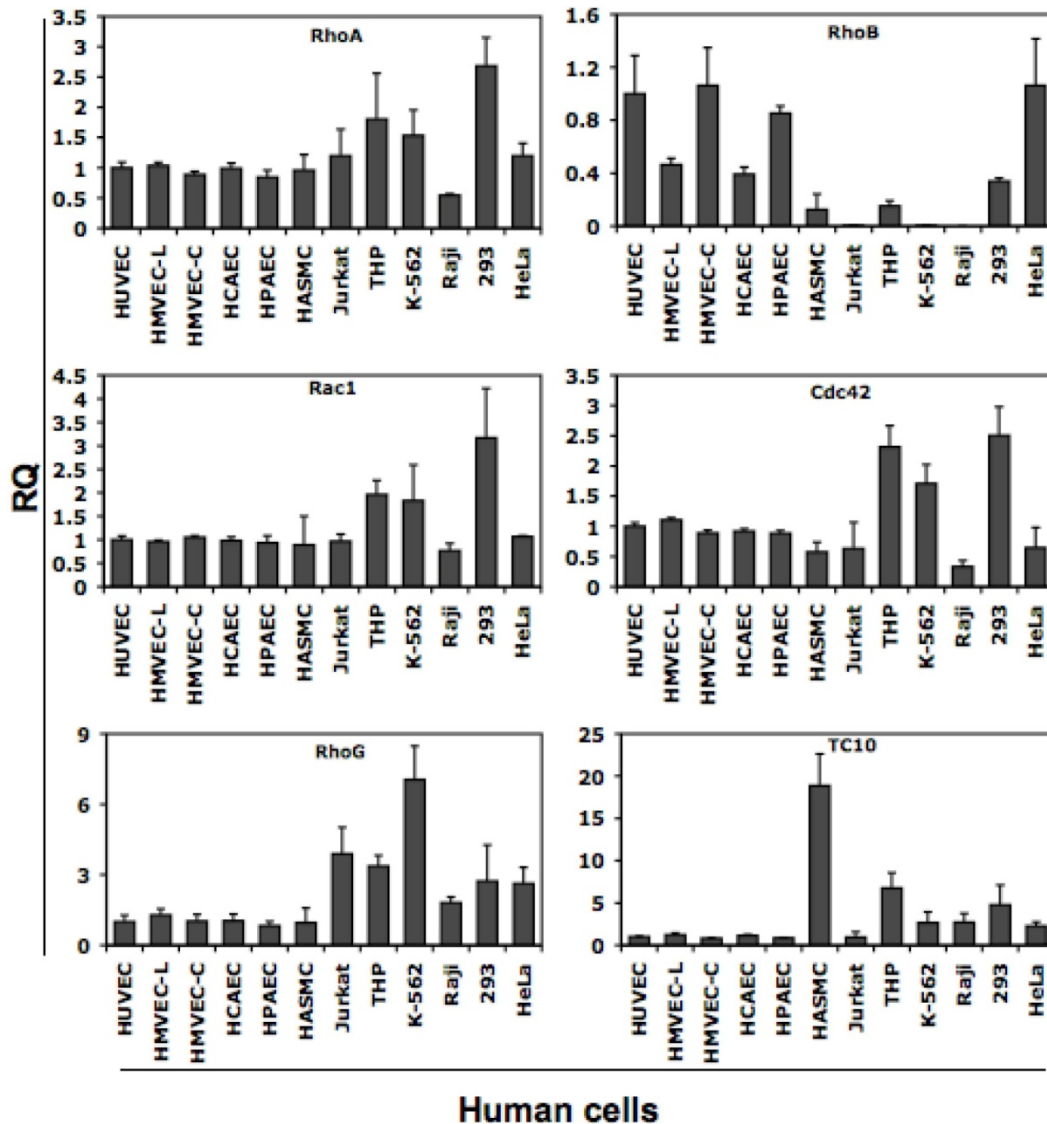
(A) Expression of ERG and RhoJ during EC lumen and tube formation.

Extracts of EC cultures during EC lumen and tube formation were prepared at the indicated time points and probed for ERG, RhoJ and  $\beta$ -actin (control) in order to assess the protein expression patterns of the molecules during 3D morphogenesis. (B) Additional representative images of Western blot from Rho GTPase activity assay. EC were transfected with ERG, RhoJ or control siRNA. Cultures were established as described in Methods and after 16 hours, detergent lysates were prepared to assess the degree of Cdc42, Rac1 and RhoA activation. Starting material lysates as well as the eluates from the Pak and Rhotekin beads were assessed by Western blots using anti-Cdc42, anti-Rac1 and anti-RhoA antibodies.



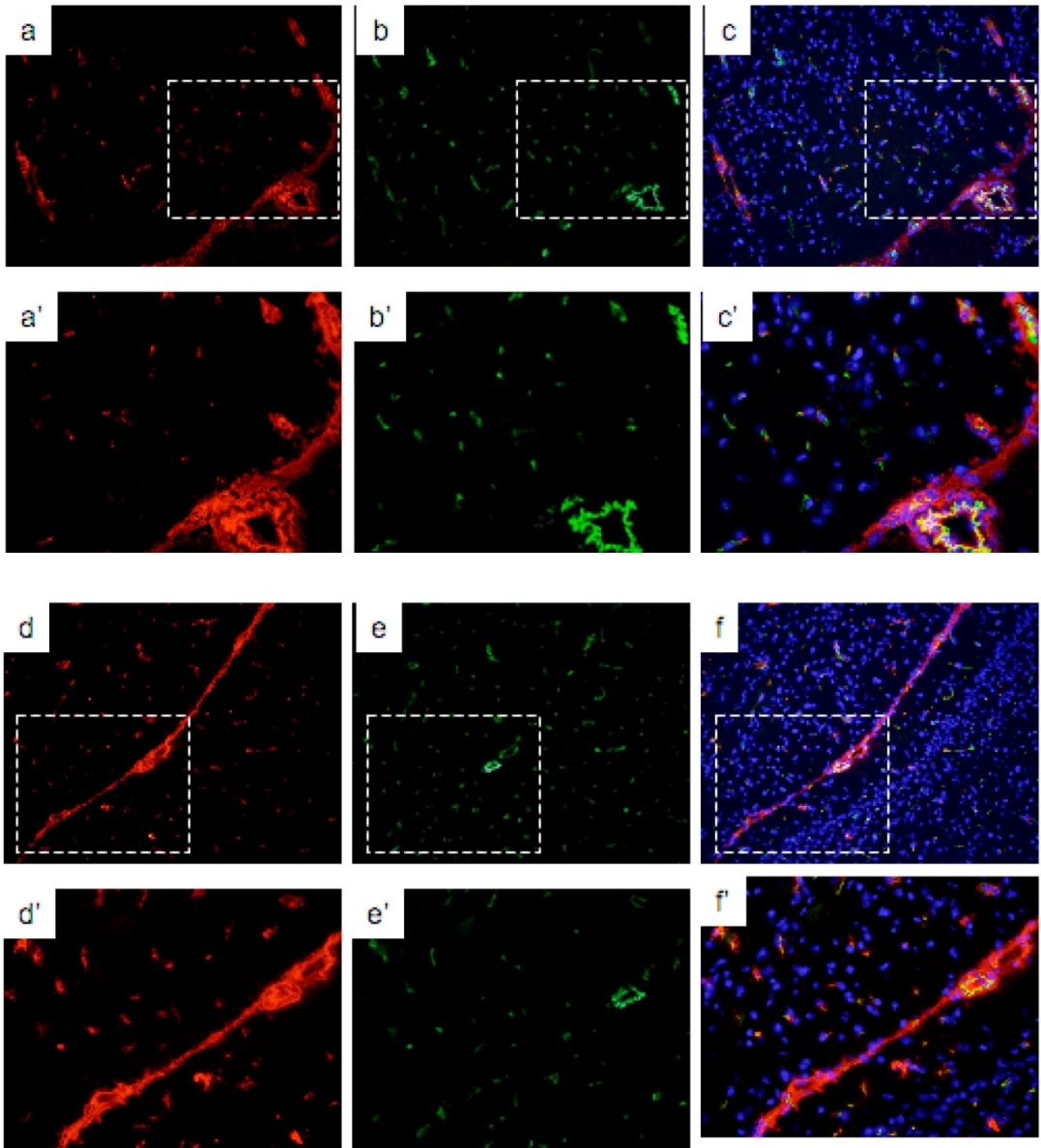
**Figure S6. Densitometric analysis**

Western blot bands were scanned from x-ray films that were exposed to blots incubated with antibodies and chemiluminescent reagents. The band intensities were quantitated using the NIH image program.



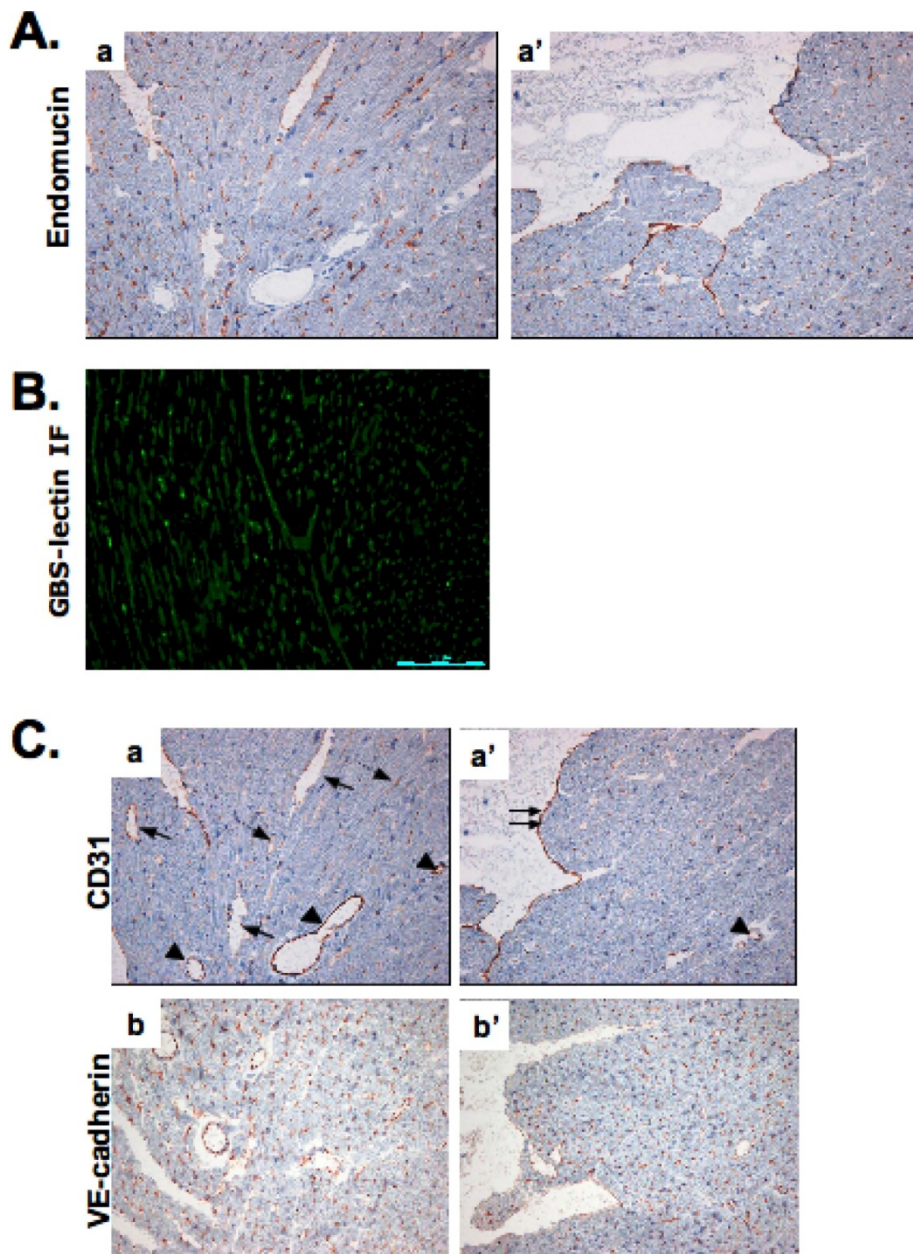
**Figure S7. Expression of selected Rho GTPases in various human cell types**

RNAs were extracted from different cell types. QPCR was performed using gene specific primers (n=3). TBP was used as an internal control for normalization.



**Figure S8. RhoJ expression in mouse brain**

Additional representative images of immunofluorescent staining of RhoJ in mouse brain under both lower- and higher-powered microscopy. Tissues were frozen-sectioned and stained for RhoJ (red), CD31 (green), or DAPI (blue). Magnification in a,b,c,d,e, and f is 20×, a',b',c',d',e', and f' is 40×.



**Figure S9. Identification of different types of ECs in the heart for laser microdissection**

(A) Representative images of Endomucin immunohistochemistry staining. Sectioned slides sequential to the laser microdissected one were stained with anti-endomucin antibody to separate arterioles from venules. (B) Representative images of immunofluorescent staining of griffonia lectin to demonstrate the location of capillaries for LMD. (C) Representative images of immunohistochemistry stained with IgG against CD31 or VE-cad for sections described above. Different ECs were annotated using arrow and arrowheads (a and a'). Solid arrow=VE (venule EC), dash arrow=CE (capillary EC), double arrow=EE (endocardial EC), and solid arrowhead=AE (artery EC). Images of a' and b' present the sections including endocardial regions.