Supporting Information

Dbouk et al. 10.1073/pnas.1419057111

SI Materials and Methods

RNA Extraction and Quantitative Real-Time PCR. Total RNA was isolated with TRI Reagent (Applied Biosystems). cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad). RNAs were quantified by RT-PCR with iTaq Universal SYBR Green Supermix (Bio-Rad) on a Bio-Rad C1000 thermocycler. Relative mRNA expression was normalized to β-actin mRNA. Transcript amounts in knockdown cells were plotted as fold change relative to control. Data were analyzed using Bio-Rad CFX Manager (version 3.0).

Protein Extraction and Immunoblotting. HUVECs were treated with siRNA for 48 h, lysed in Laemmli sample buffer with 0.2 M DTT and 10% (vol/vol) β-mercaptoethanol, resolved on 10% (vol/vol) polyacrylamide gels in SDS, and transferred to nitrocellulose membranes. Membranes were blocked for 2 h in 20% (vol/vol) LI-COR blocking buffer in Tris-buffered saline, 0.05% Tween 20 (TBST), and incubated overnight with primary antibodies at 4 °C. Membranes were washed with TBST and incubated for 1 h at room temperature with the secondary antibodies donkey anti-rabbit IRDye 680RD or donkey anti-mouse IRDye 800CW (1:10,000; LI-COR Biosciences). Membranes were washed with TBST and imaged using the LI-COR Odyssey Infrared imaging system. Blots were quantified using LI-COR Odyssey application software (version 3.0).

ELISA. HUVECs were treated with siRNAs for 48 h in six-well dishes and starved for 2 h in conditions replicating those before cord formation. Supernatants were flash-frozen at −80 °C and thawed on ice. Secreted VEGF-A was measured in 100 μL with the VEGF-A ELISA Kit (Thermo Scientific). Amounts were calculated as absorbance at 450 nm minus that at 550 nm using the Synergy 2 multimode microplate reader (BioTek) with Gen5 software.

Bead Sprouting Assays. Endothelial cells were incubated overnight with Cytodex 3 (Fisher Scientific) at a ratio of 400 cells per bead. After overnight incubation, cell-coated beads were mixed with a 2 mg/mL fibrinogen (Sigma-Aldrich) solution and pipetted into 24-well dishes containing 12 μL 50 U/mL thrombin (Sigma-Aldrich) and allowed to polymerize for 30 min before plating 10,000 primary human lung fibroblasts (ATCC) on top. The medium was changed every other day, and cells were imaged after 2–3 wk using a microscope (Axiovert 200M; Carl Zeiss) with a 10× objective lens and equipped with a digital camera (SensiCam; Cooke).

Spheroid Sprouting Assays. HUVECs were counted and 8,000 cells were plated in 200 μL in a 96-well low-adhesion plate (Costar) for 24 h to form spheroids. After 24 h, spheroids were plated in 3.2 mg/mL neutralized bovine collagen I (Corning) in a 96-well plate and imaged 24 h later using a microscope (Axiovert 200M; Carl Zeiss) with a 10× objective lens and equipped with a digital camera (SensiCam; Cooke).

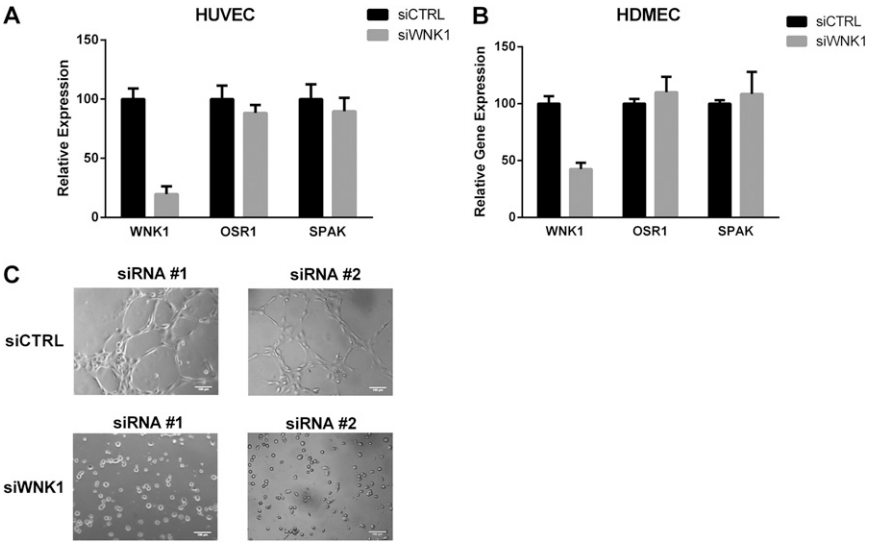

Fig. S1. Specificity of WNK1 siRNAs. (*A* and *B*) Amounts of mRNAs encoding WNK1, OSR1, and SPAK in (*A*) HUVECs and (*B*) HDMECs relative to β-actin measured by qRT-PCR. (*C*) Effect of WNK1 depletion using two different siRNAs (siWNK1 1 is shown in Fig. 1*D*; siWNK1 2 is a different siRNA) on cord formation by HUVECs. (Scale bars, 100 μm.)

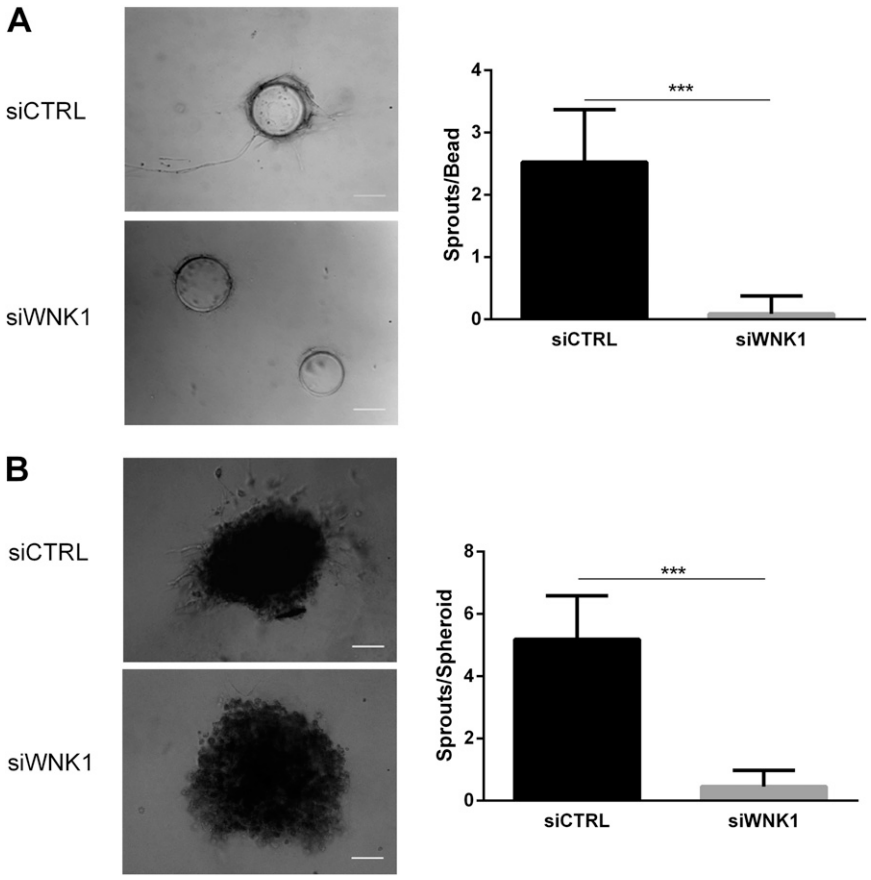

Fig. S2. Effect of siWNK1 on endothelial sprouting. (*A*) Effect of WNK1 depletion on angiogenic sprouting of HDMECs in bead sprouting assays, and quantification of the number of sprouts per bead. (*B*) Effect of WNK1 depletion on sprouting in HUVECs in a spheroid sprouting assay, and quantification of the number of sprouts per spheroid. Error bars are standard deviation. Data show representative images from three experiments (*B*) and two experiments (*A*). ***$P < 0.001$. (Scale bars, 100 μm.)

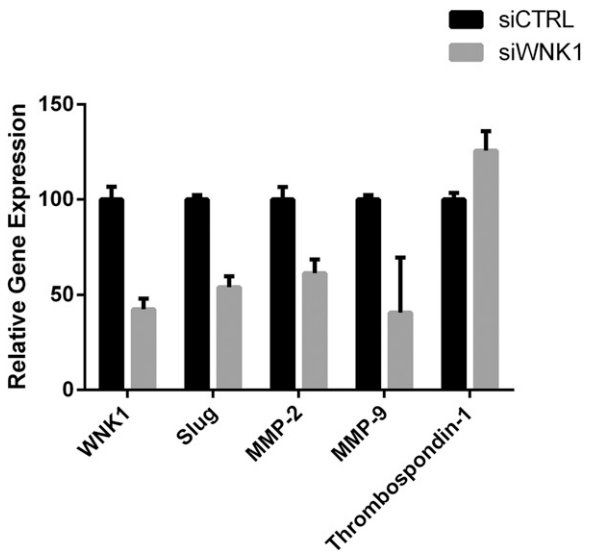

Fig. S3. WNK1 affects EMT genes in HDMECs. Changes in amounts of EMT-related genes in HDMECs following treatment with the indicated oligonucleotides, measured by qRT-PCR. Data are mean ± SEM from two experiments.

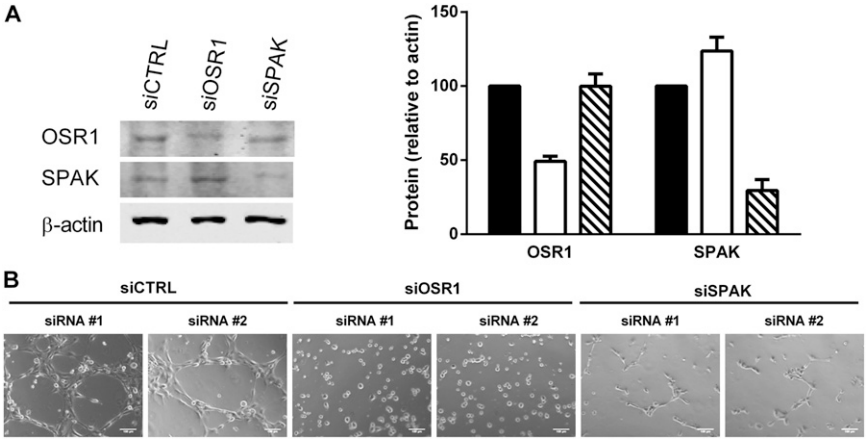

Fig. S4. Confirmation of OSR1 and SPAK siRNAs. (*A*) Whole-cell lysates from HUVECs treated as indicated were immunoblotted with anti-OSR1, anti-SPAK, and anti–β-actin as a loading control. Blots were quantified via a LI-COR Odyssey imaging sytem. Data are mean ± SD from two experiments. (*B*) Effect of OSR1 or SPAK depletion (siOSR1 1 and siSPAK 1 are shown in Fig. 3*A*; siOSR1 2 and siSPAK 2 are different siRNAs) in HUVECs, using two different siRNAs, on cord formation. (Scale bars, 100 μm.)

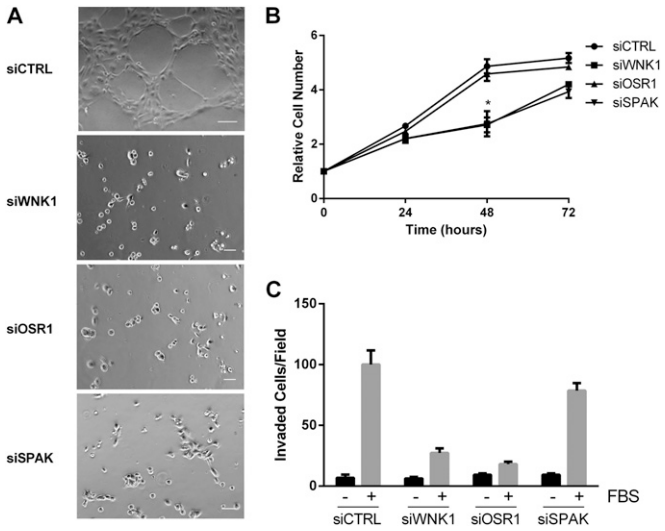

Fig. S5. OSR1 and SPAK impact HDMEC cord formation, proliferation, and invasion. (*A*) Effect of WNK1, OSR1, or SPAK depletion on HDMEC cord formation. (Scale bars, 100 μm.) (*B*) Proliferation of WNK1-, OSR1-, or SPAK-depleted HDMECs over a 72-h time course. Cell numbers are shown relative to those at 0 h. (*C*) Migration of WNK1-, OSR1-, or SPAK-depleted HDMECs through a membrane toward media without or with 10% (vol/vol) FBS. Data are mean ± SEM from three experiments (*B* and *C*); representative images are from three experiments (*A*). $^*P < 0.05$. (Scale bars, 100 μm.)

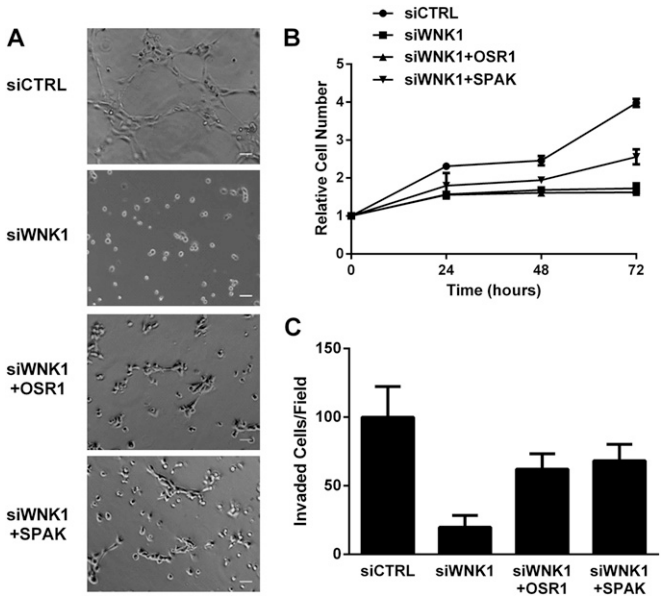

Fig. S6. Rescue of siWNK1 HUVECs with overexpressed OSR1 or SPAK. (*A*) Effect of overexpression of OSR1 or SPAK in WNK1-depleted HUVECs on cord formation. (Scale bars, 100 μm.) (*B*) Proliferation of HUVECs with the indicated treatments over a 72-h time course. Cell numbers are shown relative to those at 0 h. (*C*) Migration of HUVECs with the indicated treatments through a membrane toward media with 10% FBS. Data are mean ± SD from two experiments (*B* and *C*); representative images are from two experiments (*A*). (Scale bars, 100 μm.)