Supporting Information

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SI Materials and Methods

Materials. Mouse monoclonal antibodies (mAbs) against VE-cadherin (sc-9989) and rabbit polyclonal antibodies (pAbs) against Smad1/5 (sc-6031) were purchased from Santa Cruz Biotechnology. Mouse mAbs against GAPDH (MAB374) were purchased from Millipore. Mouse mAbs against Fer (#4268) and rabbit pAbs against phospho–β-catenin [serine 45/threonine 41 (Ser45/Thr41); #9565], phospho–β-catenin (S33/37) (#2009), and connexin 43 (#3512) were purchased from Cell Signaling Technology. Rabbit pAbs against phospho–β-catenin (Tyr142; #CP1081) were purchased from ECM. Mouse mAbs against human β-catenin (610154), N-cadherin (610921), and bone morphogenetic protein receptor-II (BMPRII; 612292) were purchased from BD Transduction Laboratories. Mouse mAb against histone H1 (#05–457) was purchased from Upstate. Rabbit pAb against Fyn (GTX101189) was purchased from Gene Tex. Rabbit pAb against phospho-Fer (Tyr402) (AB79573) was purchased from Abcam. The control siRNA and specific siRNAs for Cx43, Fer, Fyn, N-cadherin, VE-cadherin, BMPRII, Smad1, and Smad5 were purchased from Invitrogen. Noggin (3344-NG-050) was purchased from R&D Systems. All other reagent-grade chemicals were obtained from Sigma unless otherwise noted.

DNA Constructs and Mutagenesis. A PCR-amplified human β-catenin cDNA was cloned into pCMV-3Myc (Stratagene) between BamHI and SalI. The PCR primer sequences used were as follows: sense, 5′-AAAGGATCCAGCGTGGACAATGGCT-3′; antisense, 5′-CCG GGTCGACAGCTAAAGGATGATTTACAG-3′. Single-point mutations resulting in codon changes from tyrosine to alanine or glutamate for residue Tyr142 or from serine to alanine or aspartate for residue Ser45 were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) on the Myc–β-catenin plasmid. The mutagenic sense primers used were as follows (underline indicates the mutation site): 5′-GTAAACTTGATTAACGCTCAAGATGATGCAGAACTTGCC -3′ (Y142A), 5′-GTAAACTTGATTAACGAGCAAGATGATGCAGAACTTGCC-3′ (Y142E), 5′-CACTACCACAGCTCCTGCTCTGAGTGGTAAAGG-3′ (S45A), and 5′-CAC TACCACAGCTCCTGATCTGAGTGGTAAAGG-3′ (S45D). All plasmids were verified by DNA sequencing. Bovine aortic endothelial cells (BAECs) were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

Transfections. For plasmid transfections, cells at 60% confluence were transfected with β-catenin plasmids using the Lipofectamine 2000 Transfection Reagent (Invitrogen). For siRNA transfections, cells at 70–80% confluence were transfected with the designated siRNA at 40 nM using the RNAiMax Transfection Reagent (Invitrogen).

Flouroescence Staining. ECs and smooth muscle cells (SMCs) were preloaded with CMFDA cell tracker (green) and Calcein red orange (red), respectively, and cocultured on opposite sides of 1.0-μm pore-size membrane for 24 h. Both types of cells were fixed with 4% (wt/vol) paraformaldehyde, stained with N-cadherin, mounted with ProLong Gold (Invitrogen) on glass coverslips, and photographed with a Leica TCS SP5 confocal microscope.

Transmission Electron Microscope Study. ECs and SMCs were cocultured on opposite sides of 0.02-μm pore-size membrane for 24 h, and then both types of cells were fixed with 2.5% (wt/vol) glutaraldehyde at 4 °C for 16 h and incubated with 1% osmium tetroxide for 1 h. The cells were dehydrated by graded concentrations of ethanol, embedded using Spurr's kit (Sigma) in embedding capsule, and then polymerized at 60 °C for 8 h. Sections were trimmed by ultramicrotome, mounted on the copper grides, and stained with uranyl acetate and lead citrate. The images were examined by a transmission electron microscope (Hitachi, H600).

Western Blot Analysis. ECs were collected by scraping and lysed with a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (100 μg of protein) was separated by SDS/PAGE (8% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed using the Western-Light Chemiluminescent Detection System (Applied Biosystems).

Immunoprecipitation. The cells were scraped and lysed with a buffer containing 25 mM Hepes, pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.125 M NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 mg/mL leupeptin, and 2 mM BGP. The cells were disrupted on ice by repeated aspiration through a 21-gauge needle. The same amount of protein from each sample was incubated with the designated antibody for 2 h at 4 °C with gentle shaking. The immune complex was then incubated with protein A/G plus agarose for 1 h and collected by centrifugation. These agarose-bound immunoprecipitates were washed and incubated with boiling sample buffer containing 62 mM Tris·HCl, pH 6.7, 1.25% (wt/vol) SDS, 10% (vol/vol) glycerol, 3.75% (vol/vol) mercaptoethanol, and 0.05% (wt/vol) bromophenol blue. The samples were then subjected to SDS/PAGE and Western blotting.

RNA Isolation and RT-PCR. ECs in coculture were collected by scraping, and total RNA from each cell type was isolated by the guanidium isothiocyanate/phenochloroform method and converted to cDNA using the SuperScript II Reverse Transcriptase System and oligo-dT primers (Life Technologies). Briefly, total RNA [2 μg in diethyl pyrocarbonate (DEPC) water] was incubated with 50 U/μL SuperScript II RNase H-Reverse Transcriptase in buffer containing 20 mM Tris·HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.5 mM deoxynucleoside triphosphate (dNTP) mix, 10 mM DTT and oligo-$dT_{12–18}$ (0.5 μg/mL) for 50 min at 42 °C. Reactions were terminated with *Escherichia coli* RNase H (2 U/μL). The cDNA was diluted 1:20 before the conduction of PCR with 1 μL cDNA in 20 mM Tris·HCl, pH 8.4, 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM sense and antisense primers, and Taq polymerase (2 U/μL; Takara Shuzo). The PCR cycles for each reaction were as follows: heat denaturation at 94 °C for 1 min, primer annealing at 60 °C for 2 min, and primer extension at 72 °C for 2 min. Primer sequences were as follows: intercellular adhesion molecule-1 (ICAM-1) (sense, 5′-GGTGAATTGCACGCTGGATG-3′, antisense, 5′-ATATTCTGGCCGTGGAGC AC-3′, product length of 90 bp); vascular cell adhesion molecule-1 (VCAM-1) (sense, 5′-CTATGAGGGCAGAAG ATTCTGG-3′, antisense, 5′-AATTGGTAACCGAGCCAGAC-3′, product length of 441 bp); and E-selectin (sense, 5′-GTGTCACAGTATCTCTCTGC-3′, antisense, 5′-GGCACTGACA AGTGTAGTTG-3′, product length of 185 bp). cDNA amplifications were performed in parallel samples using bovine β-actin primers (sense, 5′-ATTGAGCACGGCATCGTCAC-3′, antisense, 5′-TTGCCGATGGTGATGACCTG-3′, product length of 599 bp). The PCR

reactions were carried out using the GeneAmp System 9700 (PE Biosystems). The amplified cDNAs were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Band intensities were quantified directly from the stained agarose gels using video imaging and a densitometry software system (GDS-8000 Imaging Workstation, UVP, Inc.).

Permeability Assay. ECs were seeded on fibronectin-coated Transwell filters in six-well dishes and cultured with 2 mL Medium 199 with 20% (vol/vol) FBS in the upper chamber and 1 mL growth medium in the lower chamber. The cells were grown overnight, after which the medium in the upper chamber was changed to fresh F12K medium with 10% FBS or SMC-conditioned medium (CM) and was then incubated for 18 h. For the assay, 40 μL of FITC-dextran (*M*r 38900, Sigma, final concentration of 1 mg/mL) were added to the upper chamber. After 25 min of incubation, the medium was removed from the lower compartment and diluted with PBS. Fluorescence was measured at the 492/520 nm absorption/emission wavelengths for FITC-dextran.

Monocyte Adhesion Assay. Monocytic THP-1 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Gibco) culture medium supplemented with 10% (vol/vol) FBS. THP-1 cells were labeled with calcein acetoxymethyl ester (calcein-AM; Molecular Probes) at a concentration of 7.5 μM for 30 min immediately preceding the adhesion assays. The labeled THP-1 cell suspension (5×10^5 cells/mL) was perfused over the EC side of the coculture for 30 min. Nonadherent cells were removed by washing with buffer, and the adherent THP-1 cells on the ECs were identified and counted in 20 or more randomly selected microscopic fields (640 μm × 480 μm) under a 20× objective (NA = 0.4, LD Achroplan; Zeiss).

Statistical Analysis. Results are given as mean ± SEM. Statistical tests were performed using an independent Student *t* test for the two groups of data and an ANOVA followed by the Scheffé's test for multiple comparisons. A P value < 0.05 was considered to be significant.

SI Results

VE-cadherin is responsible for the integrity of homocellular junctions and regulates the EC barrier function. We determined whether the β-catenin–Ser45/Thr41 phosphorylation induced by the SMC paracrine effect modulates EC permeability. ECs were treated with SMC-CM for 18 h; FITC-dextran was then added and the cells were incubated for additional 25 min. SMC-CM increased the EC permeability to FITC-dextran by ~2.2-fold (Fig. S3*A*). As positive controls, the transwells with TNF-α–treated ECs or without ECs showed increases of ~2.8-fold. The increased permeability of ECs was reduced by their transfection with BMPRII- and Smad5-specific siRNAs (Fig. S3*B*). Transfecting BAECs with a dominant-negative mutant of β-catenin–Ser45 (S45A) blocked the increase in FITC-dextran permeability induced by SMC-CM (Fig. S3*C*). Transfecting BAECs with a dominant-positive mutant of β-catenin–Ser45 (S45D) increased the FITC-dextran permeability of BAECs (~1.8-fold) in the absence of bovine aortic SMC-CM stimulation (compared with the empty vector-transfected monocultured BAECs).

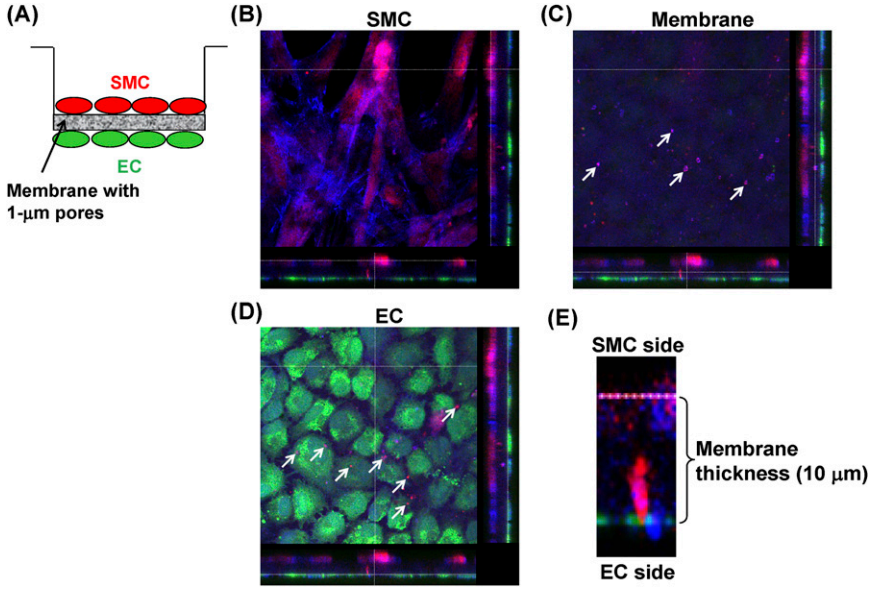

Fig. S1. SMCs penetrate through the 1-μm pores of the coculture membrane to contact with ECs. ECs and SMCs were labeled with CMFDA cell tracker (green) and Calcein red orange (red) (Molecular Probes), respectively, and then cocultured on opposite sides of 1-μm pore-size membranes for 24 h (*A*). The EC/SMC coculture was then immunostained with N-cadherin (blue), which is specific to SMCs, but not ECs. The EC/SMC coculture was serially scanned under confocal microscopy (Leica TCS SP5) using a 63×-oil objective from the inner (*Upper*) side of the membrane where SMCs were seeded (*B*), through the membrane region (*C*), to the outer (*Lower*) side of the membrane where ECs were seeded (*D*). *E* is a representitive image of 3D reconstruction from the sequential *z*-stacks of images showing a SMC penetrating through the membrane to make contact with an EC. The arrows in *C* and *D* indicate the 1-μm pores through which SMCs penetrate to the EC side (*D* and *E*). ECs were not able to migrate through the membrane to reach the SMC side.

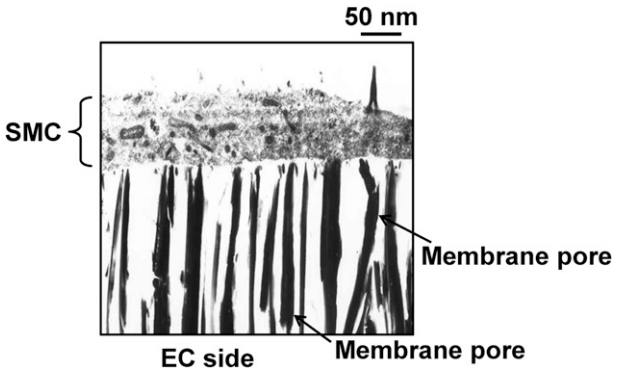

Fig. S2. SMCs in EC/SMC coculture cannot penetrate into the 0.02-μm pores of coculture membrane. ECs and SMCs were cocultured on opposite sides of a membrane with 0.02-μm pores for 24 h and then fixed with 2.5% (wt/vol) glutaraldehyde, embedded in Spurr's solution, and stained with uranyl acetate and lead citrate. The specimens were examined by transmission electron microscopy (Hitachi, H600) with 8,000× magnification. The image shows that the SMCs in EC/SMC coculture cannot penetrate into the 0.02-μm pores of the coculture membrane. (Scale bar, 50 nm.)

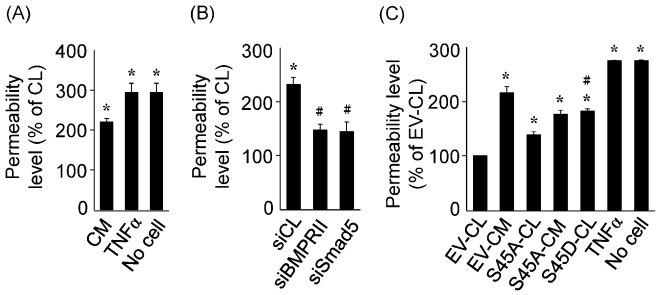

Fig. S3. Ser45/Thr41-phosphorylation of β-catenin in ECs induced by paracrine interaction with SMCs is critical for modulating EC permeability. (*A*) ECs were kept as control, treated with SMC-CM for 18 h, or activated with TNF-α for 4 h. (*B*) ECs were transfected with control, BMPRII-, or Smad5-specific siRNA. (*C*) ECs were transfected with an control empty vector or β-catenin mutants (S45A or S45D) and then kept as controls or treated with SMC-CM for 18 h. EC permeability was determined as described in *SI Materials and Methods*. The results are the mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. unstimulated control cells. $^{\#}P < 0.05$ vs. cells transfected with control siRNA or empty vector.

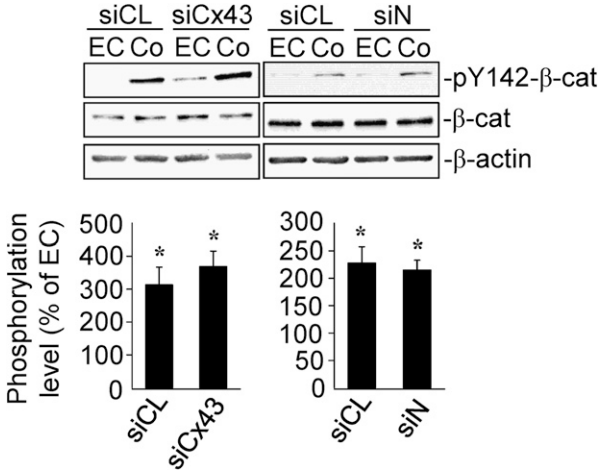

Fig. S4. The Tyr142-phosphorylation of β-catenin in ECs induced by their contact with SMCs is not regulated by SMC Cx43 and N-cadherin. SMCs were transfected with control- (siCL), Cx43- (siCx43), and N-cadherin-specific siRNAs (siN) for 24 h, and then cocultured with ECs for 18 h (Co). ECs kept as a monoculture were used as controls. The Tyr142-phosphorylation of β-catenin in ECs was determined by Western blot analysis. The results are the mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. monocultured ECs.