Supporting Information

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SI Materials and Methods

Animals. Male C57BL/6J (age ~6–8 wk; $n = 107$; stock number: 000664) and B6.FVB-Tg (ITGAM-DTR/EGFP) 34Lan/J (age ~6–8 wk; $n = 113$, stock number: 006000) mice were purchased from Jackson Laboratory. The FVB-Tg (ITGAM-DTR/EGFP) 34Lan/J transgenic mice encode a diphtheria toxin (DT) receptor fused with GFP under the control of a human integrin αM (ITGAM) promoter (CD11b). The transgenic mice provide a DT-inducible system for transient depletion of macrophages (1–4). The C57BL/6J mice served as controls. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University (IACUC approval number: B11265).

Primary Cell Culture. The cochleae from 7- or 10-d-old C57BL/6J mice ($n = 40$) were harvested under sterile conditions. The stria vascularis was pulled gently away from the spiral ligament, placed in ice-cold Hank's calcium- and magnesium-free balanced salt solution, and minced into small pieces (about 1-mm^3) with ophthalmic tweezers. To produce endothelial cells (ECs), the minced stria vascularis was cultured on collagen-coated dishes in CSC medium (cat# C1556-100ML, Sigma) containing 1% endothelial cell growth factor, 10% (vol/vol) FBS with 100 U/mL penicillin G, and 100 μg/mL streptomycin. To produce perivascular-resident macrophage-like melanocytes (PVM/Ms), the minced stria vascularis was cultured on collagen-coated dishes in medium 254 (Invitrogen) containing 1% human melanocyte growth factor, 10% FBS, and 0.5% gentamicin/amphotericin. The minced stria vascularis was incubated at 37° in 5% CO_2 with the medium changed every 3 d. Cell clones formed and melted at about 10 d. The cells were detached from the cell colony with a solution of trypsin-EDTA and then were purified. For purification of ECs, the cells were resuspended in 500 μL Isolation Buffer (calcium- and magnesium-free PBS supplemented with 0.1% BSA and 2 mM EDTA) combined with 25 μL DSB-X–labeled antibody [rat anti-mouse F4-80 (eBioscience) and rabbit polyclonal CD34 (Santa Cruz)]. For purification of endothelial cells, antibody for endothelial marker protein, CD34, was added to the medium. For purification of PVM/M cells, antibody F4/80 was added to the medium. Cells were incubated with the antibodies for 10 min at 4°, 1 mL cold isolation buffer was added for washing, and the cells were centrifuged at 300 × g for 6 min. Then 1 mL cold isolation buffer containing 75 μL FlowComp Dynabeads was added to the cell pellet (following the manufacturer's instructions) for 15 min at 4°, placed in a magnetor for 1 min, and then removed carefully. The supernatant was discarded. Isolation buffer (1 mL) was added and gently pipetted five times, and the tube was placed in the magnet for another minute. After the supernatant was discarded, 1 mL FlowComp Release Buffer was added for 10 min at room temperature and was mixed with rolling and tilting. The tube was placed in the magnet again for 1 min. The supernatant was transferred to a new tube, and the procedure was repeated. The suspension was centrifuged at 300 × g for 6 min. The supernatant was discarded, and the precipitate was suspended in 1 mL EC medium. The suspension was transferred to a 3.5-mm collagen-coated dish and incubated at 37° in 5% CO_2 for 3 d. A nonenzymatic cell dissociation solution (Sigma) was used to remove cells from the culture. Cells then were passaged at a split ratio of 1:2. ECs (passages 3–4) were grown in endothelial cell growth medium 2 at 37 °C in a 5% CO_2 incubator. The medium was changed 1 d before experimentation. PVM/Ms were grown in medium 254 containing 1% human melanocyte growth factor.

Transwell Monolayer Permeability Assay. The PVM/M contribution to EC monolayer permeability was assessed by comparing the EC-PVM/M coculture with null and EC-only monolayer (confluent) controls. The null control consisted of purified ECs at passage 3 (at a density of $4 \times 10^4/cm^2$) seeded and grown on the polyethylene terephthalate membranes of the Transwell inserts (catalog number 353096) overnight. In all cases, EC monolayers were not formed by overnight growth. The confluent EC monolayer was constructed of purified ECs of the same aliquot and of the same passage number, density seeded and grown on the inserts for 5 d. For coculture models, PVM/Ms at passage 3 (at a concentration of 3×10^4 per well) were preseeded in the lower well surface of the Transwell receiver trays (part number PIMWS2450) for 3 d before coculture with ECs and then were cocultured with ECs up to 5 d Permeability assays were performed 5 d after initial EC seeding. Chambers were examined microscopically for EC monolayer confluence, integrity, and uniformity and were assessed for presence of cell–cell tight junctional proteins including zona occludens, ZO-1, and occludin. Transendothelial permeability of FITC-dextran (molecular mass: 70 kDa) through the cell monolayers was assayed using the Millipore Vascular Permeability Assay Kit (catalog number ECM644) according to the manufacturer's instructions and as described by Lal et al. (5). Briefly, the small fluorescent molecule (diluted 1:40 with medium) permeates through the monolayer (null and EC) into the basolateral chamber (6). The amount of dextran in the basolateral chamber is directly proportional to the permeability of the endothelial cells. Permeability of the EC monolayer was measured by detecting FITC-dextran fluorescence with a multiwell microplate reader (Tecan GENios ELISA reader; Tecan Group Ltd.) at excitation/emission wavelengths of 485/535 nm. For cells treated with pigment epithelial-derived factor (PEDF), PEDF (catalog number 50235-M08H; Sino Biological Inc.) at a final concentration of 20 nM was added to the culture medium for 5 d.

In-Cell Western Blotting. Monocultured ECs or cocultured ECs at the same concentration at day 5 (5×10^5/mL, 200 μL per well) were seeded for immunohistochemical analysis in 96-well microplates and were incubated until the cells attached. The cells were fixed in 4% (wt/vol) formaldehyde for 20 min at room temperature. The cells were permeabilized with 1× PBS containing 0.1% Triton X-100, were blocked in blocking buffer for 1 h, and were incubated with primary antibodies (anti–ZO-1, anti-occludin, and anti–vascular endothelial (ve)-cadherin (see Table S1 for antibody information) for 2 h at 4 °C. Then the cells were washed four times for 5 min each washing in PBS containing 0.1% Tween-20 and were incubated with 1:100 secondary antibodies including goat Alexa Fluor 680, goat anti-rabbit IgG, and goat anti-mouse IgG (LI-COR Bioscience) for 1 h at room temperature. Cells were washed again three times in PBS. Stained cells were imaged and analyzed with an Odyssey Imager (Li-COR Biosciences).

Immunohistochemistry and Fluorescence Microscopy. The cochleae were isolated, harvested, fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, and rinsed in 37 °C PBS (pH 7.3) to remove any residual 4% PFA. Immunohistochemistry was performed as described previously (7). Tissue samples were permeabilized in 0.5% Triton X-100 (Sigma) for 1 h and were immunoblocked with a so-

lution of 10% (vol/vol) goat serum and 1% bovine albumin in 0.02 mol/L PBS for an additional hour. The specimens were incubated overnight at 4 °C with the primary antibodies (listed in Table S1) diluted in PBS-BSA. After several washes in PBS, the samples were incubated with the secondary antibodies [Alexa Fluor 568-conjugated goat anti-rabbit (catalog number A21069l; Invitrogen), Alexa Fluor 647-conjugated goat anti-rabbit (catalog number A-21244l; Invitrogen), or Alexa Fluor 568-conjugated goat anti-rat (catalog number A-11077l; Invitrogen)] for 1 h at room temperature. The tissues were mounted in mounting medium (H-1000; Vector Laboratories) and visualized under an FV1000 Olympus laser-scanning confocal microscope. Controls were prepared by replacing primary antibodies with PBS. 3D rendering of confocal data was performed using the image processing pack Fiji (http://fiji.sc/wiki/index.php/Fiji) and UCSF Chimera (Resource for Biocomputing, Visualization, and Informatics; http://www.cgl.ucsf.edu/chimera/) for visualization, processing, analysis, and presentation. Individual channels were split and converted into separate image stacks, which then were filtered to reduce the signal-to-noise ratio. Volume data then were displayed at respective thresholds as solid or partially transparent isosurfaces in UCSF Chimera using the volume viewer.

ELISA. The concentration of PEDF in the PVM/M culture medium was measured by an ELISA using commercially available reagents (catalog number E91972Mu; Uscn life Science Inc.) according to the manufacturer's instructions.

Assessment of Vascular Permeability. Vascular permeability in control and DT-treated mice was compared using various tracers, including Evans blue (molecular mass 961 Da) (catalog number E2129; Sigma Aldrich), lysine-fixable cadaverine conjugated to Alexa Fluor-555 (molecular mass 950 Da) (catalog number A-30677\; Invitrogen), BSA-conjugated to Alexa Fluor-555 (molecular mass 66K Da) (catalog number A-34786; Invitrogen), and goat anti-human IgG conjugated to Alexa Fluor-568 (molecular mass 200 kDa) (catalog number A-21090; Invitrogen). The tracers were i.v. injected into the tail vein of anesthetized control and drug-treated animals for 3 min. Anesthetized animals were perfused intravascularly through the left ventricle with HBSS, pH 7.4, followed by 4% (wt/vol) PFA as a fixative. The mice were decapitated, and their cochleae were harvested immediately. Whole-mounted cochlear lateral wall tissue from each tracer group (cohorts of three) was dissected carefully. Fluorescent images of dissected lateral wall were captured using a fluorescent microscope (Leica DM2500, equipped with an HBO 100 lamp and Leica DFC 420 C digital camera).

For the quantitative analysis, mice in DT-treated and control groups (three mice per group) received i.v. injections of cadaverine-Alexa Fluor-555– and Alexa Fluor-568–conjugated goat anti-goat IgG. Two hours following injection of cadaverine-Alexa Fluor-555 and 16 h following injection of Alexa Fluor-568–conjugated goat anti-goat IgG, the mice were anesthetized and perfused for 5 min with HBSS, and the cochlear lateral wall was removed and homogenized in 1% Triton X-100 in PBS, pH 7.2. The lysate was centrifuged at 12960 × *g* for 20 min, and relative fluorescence of the supernatant was measured on a Tecan GENios Plus microplate reader (Tecan Group Ltd). The samples were run in quintuplicate for each group.

For in situ detection of fluorophore-conjugated tracers, anesthetized animals were perfused for 1–2 min with HBSS, followed by a 5-min perfusion of 4% PFA in PBS, pH 7.2. Cochlear lateral wall tissue was removed and postfixed in 4% PFA in PBS, pH 7.2, at 4 °C, overnight. Whole-mounted cochlear lateral wall brain slides were immunostained with anti-collagen IV antibody (see Table 1). Samples were analyzed with an FV1000 Olympus laser-scanning confocal microscope.

Conventional Transmission Electron Microscopy. Cochlear lateral wall tissues were dissected, fixed overnight with phosphate-buffered 3% (vol/vol) glutaraldehyde-1.5% (wt/vol) PFA, and postfixed in 1% osmium. Tissues were dehydrated and embedded in Araldite plastic, sectioned, stained with lead citrate and uranyl acetate, and viewed in a Philips EM 100 transmission electron microscope.

Isolation of Stria Vascularis Capillaries. Tight-junction and adherens-junction analysis required an isolation procedure to minimize tight junction-associated protein contamination from adjoining marginal and basal cell layers. For capillary isolation, we used a previously developed and reported sandwich-dissociation method (7). Briefly, the auditory bulla from control and DT-treated groups (three mice for each group) were dissected after cardiovascular perfusion with saline, removed rapidly, and placed in a Petri dish filled with a physiological solution containing 125 mmol/L NaCl, 3.5 mmol/L KCl, 1.3 mmol/L $CaCl_2$, 1.5 mmol/L $MgCl_2$, 0.51 mmol/L NaH_2PO4, 10 mmol/L Hepes, and 5 mmol/L glucose at pH 7.4 with osmolarity adjusted to 310 mmol/kg. Each turn of the bony cochlear lateral wall containing the spiral ligament and stria vascularis was separated from the organ of Corti, and the stria vascularis was peeled away from the spiral ligament gently with Dumont tweezers (110 mm, 0.1 × 0.06 mm tip) and a Tungsten dissecting probe (50 mm, 0.5-mm diameter rod) under an Olympus SZ61dissecting microscope. The stria vascularis was placed in a glass-bottomed microwell dish (dish diameter: 35 mm; microwell diameter: 14 mm; coverglass: 0.16–0.19 mm) (MatTek Corp.) filled with the physiological solution. A glass coverslip (0.16 mm) then was positioned over the stria vascularis, and the tissue was sandwiched gently between the glass surfaces. Gentle pressure was applied to compress the stria vascularis against the two glass surfaces. By repeating this step, the nonvascular tissues were dispersed into the solution and separated from the capillaries. Nonvascular cells were flushed away gently with a 100-μL micropeptide; the clean microvessels adhered and were "printed" onto the bottom of the dish, analogous to the offset-printing deposit of a pattern of ink onto paper. The isolated capillaries then were transferred to a microfuge tube for quantitative real-time PCR or immunohistochemistry.

Reverse Transcription-Polymerase Chain Reaction. Total RNA from either isolated and purified PVM/Ms or cultured and purified PVM/Ms was extracted separately with a RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's suggestions. One microgram of total RNA was reverse-transcribed using a RETROscript kit (Ambion, Austin, TX). Conserved regions spanning introns were selected for the primers of Kcnj10 (Kir 4.1), Emr1 (F4/80), Mgst1 (GST), Serpinf1 (PEDF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used were as follows: Kcnj10 (mouse Chr 1 NM_001039484), forward, CCTCATTGGCTGCCAGGTGACA, reverse, GTCCTTCCTTTTCAGCTTGCTC, 487-bp product; Emr1 (mouse Chr 17 NM_010130), forward, tgcatctagcaatggacagc, reverse, gccttctggatccatttgaa, 170-bp; Mgst1 (mouse Chr 6 NM_019946), forward, cgccaccaaatatgacctct, reverse, cctgttgcccacaaggtagt, 232-bp; Serpinf1 (mouse Chr 11 NM_011340), forward, GGCAGTGGGTAACCAAGTTTG, reverse, GCAGCTGGGCAATCTTGCAG, 156-bp; gaphd (mouse Chr 6 NM_008084), forward, ATGTGTCCGTCGTGGATCTGAC, reverse, AGACAACCTGGTCCTCAGTGTAG, 132-bp product. The RT-PCR was cycled at 95 °C for 2 min, up to 40 cycles at 95 °C for 30 s, 60 °C for 45 s, 72 °C for 30 s, and a final 5-min extension at 72 °C. The products of RT-PCR were visualized by agarose gel electrophoresis.

Quantitative Real-Time PCR. The procedure for quantitative real-time PCR was described previously (7). For the in vitro models, total RNA from monocultured ECs and cocultured ECs was extracted

separately using RNeasy (QIAGEN). One thousand nanograms of total RNA and 100 ng of random hexamer were used to make 20 μL of cDNA by SuperScript II (Invitrogen) following the manufacturer's instructions. For the in vivo models, total RNA from the stria vascularis capillaries of control and DT-treated groups was extracted separately using RNeasy (QIAGEN). Each group of three mice was analyzed for mRNA levels of *Zo1, Occludin*, and *VE-cadherin* with quantitative real-time PCR. One microgram of total RNA and 100 ng of random hexamer were used to make 20 μL of cDNA by SuperScript II (Invitrogen) following the manufacturer's instructions. The cDNA synthesized from total RNA was diluted twofold with DNase-free water. Transcripts were quantitated using a corresponding TaqMan gene-expression assay: ZO1 (catalog number Mm00493699_m1; Applied Biosystems), occludin (catalog number Mm00500912_m1), and ve-cadherin (catalog number Mm03053719_s1) on a model 7300 real-time PCR system (Applied Biosystems). The PCR was cycled with an initial hold of 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Mouse Gapdh (catalog number 4352339E; Applied Biosystems) expression was used as an endogenous control. The samples were run in triplicate for each gene. Quantitative PCR was performed according to the guidelines provided by Applied Biosystems. A comparative cycle threshold (C_T) method ($\Delta\Delta C_T$ quantitation) was used to calculate the difference between samples. The data were analyzed according to the manufacturer's suggestions.

Immunolabeling of Isolated Capillaries. Capillaries of the stria vascularis from different animal groups were isolated with the sandwich-dissociation method described above (7). Gain in immunofluorescence in the isolated capillaries was calculated as $(F_T - F_B)/(F_C - F_B) \times 100\%$, where F_T is the average fluorescence intensity in treated groups, F_C is the average fluorescence intensity in control groups, and F_B is the background fluorescence.

Transfection with siRNA. *Pedf* silencing in vitro was performed on passage-3 PVM/Ms seeded in 24-well plates. The PVM/Ms (1×10^5 cells per well) were transfected with *Pedf* and scrambled siRNA (Applied Biosystems) used according to the manufacturer's guidelines. In brief, 2.5 μL TransIT-TKO reagent was mixed with 50 μL serum-free medium in a sterile tube. After incubation for 15 min, 25 nM siRNA was added to the mixture for another 15 min. Then the TransIT-TKO Reagent/siRNA mixture was added to the 24-well plates. Total RNA was extracted on the third day after transfection, and the *Pedf* mRNA level was detected by quantitative PCR.

For the in vivo siRNA transfection, animals were anesthetized, and a 30-G needle was used to make a single puncture in the anterior-inferior quadrant of the tympanic membrane to allow exit of air from the middle ear during drug injection. *Pedf* was silenced with a 5-μL solution of siRNA (20 ng/μL) injected through the posterior-inferior quadrant. Following the procedure described in Kaur et al. (8), the middle ear was filled completely with the solution for 5 d ($n = 3$ mice per group). Scrambled siRNA of the same concentration was given to control group ($n = 3$ mice per group). Animals were killed, and cochleae were dissected. Total RNA was extracted from the lateral wall tissue (500–700 ng) of each group, and the mRNA level of *Pedf* was detected by a quantitative PCR kit used according to the guidelines provided by Applied Biosystems. The comparative C_T method ($\Delta\Delta T$ quantitation) was used to calculate the difference between samples.

PVM/M Counts. PVM/Ms labeled with antibody for F4/80 in the stria vascularis of control and drug-treated mice (cohorts of three mice) were counted on a standard epifluorescence microscope with a 20× objective. Counts were obtained at 10 randomly chosen, nonoverlapping 150 × 300 μm areas of each group. Data are presented as mean ± SD.

Drug Treatment. Mice were assigned randomly to receive DT or control (saline) injections. DT was injected i.p. at a dose of 20 ng/g body weight on day 1 and at 10 ng/g body weight on days 3 and 4. An equal volume of saline was administered to the control mice. For the PEDF treatment, mouse PEDF protein (catalog number 50235-M08H; Sino Biological Inc.) was injected i.v. into the tail vein of the B6.FVB-Tg mice (10 μg/100 g body weight) every day for parallel DT treatment up to 5 d (9–11). An equal volume of saline was administrated to the control mice.

Auditory Testing. Auditory brain-stem response audiometry to pure tones was used to evaluate hearing function. Each animal was anesthetized and placed on a heating pad in a sound-isolated chamber. The external ear canal and tympanic membrane were inspected using an operating microscope to ensure the ear canal was free of wax and that there was no canal deformity, no inflammation of the tympanic membrane, and no effusion in the middle ear. Needle electrodes were placed s.c. near the test ear, at the vertex, and at the contralateral ear. Each ear was stimulated separately with a closed-tube sound-delivery system sealed into the ear canal. The auditory brain-stem response to a 1-ms rise-time tone burst at 4, 8, 12, 16, 24, and 32 kHz was recorded, and thresholds were obtained for each ear. Threshold was defined as an evoked response of 0.2 μV. This method was used to assess auditory brain-stem response both before and immediately after exposure to noise.

Measurement of Endocochlear Potential. The endocochlear potential was recorded under general anesthesia on control ($n = 4$) and DT-treated ($n = 6$) mice. A silver–silver chloride reference electrode was placed under the skin of the dorsum. An incision was made in the inferior portion of the left postauricular sulcus, and the bulla was perforated, exposing the basal turn of the cochlea. Access to the scala media of the basal turn was obtained by thinning the bone over the spiral ligament and making a small opening with a pick. A micropipette electrode (~2 μm) filled with 150 mM KCl was advanced through the bony aperture into the spiral ligament. Entry of the electrode tip into the endolymph is characterized by transients in recorded potentials. The electrode was advanced until a stable potential was observed. The signal was amplified through an amplifier (model 3000 AC/DC differential amplifier; A-M Systems, Inc.). The DC potentials were recorded via an A-D converter (Fluke II multimeter; John Fluke Manufacturing Co., Inc).

Statistics. All experiments were performed multiple times to validate the observations, and the data are expressed as mean ± SD. Statistical analysis was conducted using a Wilcoxon signed-rank test. A 95% confidence level was considered statistically significant.

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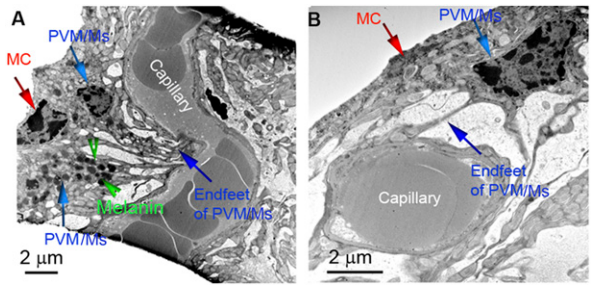

Fig. S1. The melanin-containing cells (green arrowheads) shown in the TEM images (*A* and *B*) are likely PVM/Ms. PVM/Ms in the stria vascularis are situated in close proximity to and beneath the subepithelial layer of marginal cells (MC, red arrow). The dendritic processes of PVM/Ms contact capillary wall through endfeet (dark blue arrow).

Table S1. Primary antibodies employed

Primary antibodies	Vectors	Identification	Dilution	Source	Specificity
Ve-cadherin	Abcam,	Ab33168	1:50 (dilution with 1% BSA-PBS)	Rabbit polyclonal antibody to Ve-cadherin	Reacts with Hu, Ms
F4/80	eBioscience	14-4801-85	1:50 (dilution with 1% BSA-PBS)	monoclonal antibody reacts with mouse F4/80 antigen	React with Ms
CD34	Santa Cruz	Sc-9095	1:50 (dilution with 1% BSA-PBS)	Rabbit polyclonal antibody	Reacts with Hu, Ms
ZO-1	Invitrogen	61-7300	1:25 (dilution with 1% BSA–PBS)	Rabbit polyclonal antibody	Reacts with Hu, Ms
Occludin	Abcam	ab31721	1:50 (dilution with 1% BSA-PBS)	Rabbit polyclonal antibody	Reacts with Mouse, Rat, Human, Pig
Collagen IV	Abcam	Ab6586	1:100 (dilution with 1% BSA–PBS)	Rabbit polyclonal antibody	Reacts with Mouse, Rat, Cow, Human, Pig
PEDF	Millipore	07-280	1:100 (dilution with 1% BSA–PBS)	Rabbit polyclonal antibody	Reacts with Hu, Ms
PEDFR	R&D systems	AF5365	1:100 (dilution with 1% BSA–PBS)	Sheep polyclonal antibody	Reacts with Mouse, Rat, Human