The microenvironment of the cochlea is maintained by the barrier between the systemic circulation and the fluids inside the stria vascularis. However, the mechanisms that control the permeability of the intrastrial fluid–blood barrier remain largely unknown. The barrier comprises endothelial cells connected to each other by tight junctions and an underlying basement membrane. In a recent study, we found that the intrastrial fluid–blood barrier also includes a large number of perivascular cells with both macrophage and melanocyte characteristics. The perivascular-resident macrophage-like melanocytes (PVM/Ms) are in close contact with vessels through cytoplasmic processes. Here we demonstrate that PVM/Ms have an important role in maintaining the integrity of the intrastrial fluid–blood barrier and hearing function. Using a cell culture-based in vitro model and a genetically induced PVM/M-depleted animal model, we show that absence of PVM/Ms increases the permeability of the intrastrial fluid–blood barrier to both low- and high-molecular-weight tracers. The increased permeability is caused by decreased expression of pigment epithelial-derived factor (PEDF), affects the expression of tight junction-associated proteins instrumental to barrier integrity. When tested for endocochlear potential and auditory brainstem response, PVM/M-depleted animals show substantial drop in endocochlear potential and auditory brainstem response, indicating that PVM/Ms are a hybrid cell type and differ from the classical macrophage. Given their morphological similarities to astrocytes and glial cells, we speculate the PVM/Ms might have a functional role similar to that of astrocytes in the blood–brain barrier and glial cells in the blood–retina barrier. Astrocytes and glial cells are known to have essential roles in regulation of barrier integrity and tissue-oriented functions. The hypotheses were tested in both in vitro and in vivo models. Primary endothelial and PVM/M cell lines from mouse cochlea were established for in vitro tests, and the results were validated in vivo by ablating PVM/Ms. A transgenic mouse model with a diphtheria toxin (DT) receptor under control of a human integrin αM promoter (CD11b) enabled transient depletion of the PVM/Ms. Our results demonstrate that PVM/M regulation of intrastrial fluid–blood barrier integrity, mediated by pigment epithelial-derived factor (PEDF), affects the expression of tight junction-associated proteins. The experiment provides evidence that signaling between PVM/Ms and ECs regulates intrastrial fluid–blood barrier integrity and that regulation of permeability is instrumental in maintaining a normal EP and hearing threshold.

Results

Resident Macrophages with Melanocyte Characteristics Ensheath Strial Capillaries Through Cytoplasmic Processes. Confocal images show a large population of PVM/Ms sandwiched between marginal and basal cell layers of the SV (Fig. 1A). A 3D reconstructed confocal image of the SV shows that PVM/Ms are functionally complex. The cell types of the intrastrial fluid–blood barrier have yet to be characterized functionally, and the role of PVM/Ms in the intrastrial fluid–blood barrier is particularly elusive. In an earlier study (15), we characterized the PVM/Ms as resident macrophages, because the cells are positive for several macrophage surface molecules, including F4/80, CD68, and CD11b. In this study, we further characterize the PVM/Ms as expressing several melanocyte markers. Overall, our results show that PVM/Ms are a hybrid cell type and differ from the classical macrophage. Given their morphological similarities to astrocytes and glial cells, we speculate the PVM/Ms might have a functional role similar to that of astrocytes in the blood–brain barrier and glial cells in the blood–retina barrier. Astrocytes and glial cells are known to have essential roles in regulation of barrier integrity and tissue-oriented functions. The hypotheses were tested in both in vitro and in vivo models. Primary endothelial and PVM/M cell lines from mouse cochlea were established for in vitro tests, and the results were validated in vivo by ablating PVM/Ms. A transgenic mouse model with a diphtheria toxin (DT) receptor under control of a human integrin αM promoter (CD11b) enabled transient depletion of the PVM/Ms. Our results demonstrate that PVM/M regulation of intrastrial fluid–blood barrier integrity, mediated by pigment epithelial-derived factor (PEDF), affects the expression of tight junction-associated proteins. The experiment provides evidence that signaling between PVM/Ms and ECs regulates intrastrial fluid–blood barrier integrity and that regulation of permeability is instrumental in maintaining a normal EP and hearing threshold.
situatated in or under subepithelial marginal cells (Fig. 1B, Left) but do not appear to have direct contact with basal cells (Figs. 1B, Right). The PVM/Ms are highly investated on the abluminal surface of capillaries (Fig. 1C) and are structurally intertwined with ECs with dendritic processes (Fig. 1D) that also interface with the capillary wall (Fig. S1A and B). The PVM/Ms contain melanin pigment granules (arrow). (F) Triple labeling for F4/80 (green), GSTα4 (blue), and capillaries (red) in whole-mount SV shows that PVM/Ms express GSTα4.

EC Monolayers Are Highly Permeable in the Absence of PVM/Ms. The permeability of the endothelial barrier was measured directly in a cell-culture model with an EC (Fig. 2A) and PVM/M (Fig. 2B) interface. The endothelial monolayer was verified for expression of various cell–cell tight-junction proteins, including zonula occludens 1 (ZO-1) (Fig. 2C, Left) and occludin (Fig. 2C, Right). Permeability was assessed by measuring the flux of 70-kDa fluorescent dextran across the endothelial monolayer for both the coculture model and two control models (null endothelial monolayer and confluent EC monolayer) (Fig. 2D and E). The degree of EC monolayer leakage was determined by measuring the intensity of FITC-dextran fluorescence in the basolateral chamber. Our results show that the permeability of the EC monolayer increases markedly in the absence of PVM/Ms as compared with the presence of

Fig. 1. Perivascular-resident cells identified as melanocyte-like macrophages are in close contact with capillaries through cytoplasmic processes. (A) 3D reconstructed confocal image of the SV. PVM/Ms are labeled with an antibody for F4/80 (white), and the cytoskeleton is labeled with phalloidin (red). The PVM/Ms are sandwiched between marginal (MC) and basal (BC) layers of the SV. (B) 3D reconstructed images at different angles show that PVM/Ms are situated in or under subepithelial marginal cells (Left) and have less contact with basal cells (Right). (C and D) An overview (C) and a close-up view (D) of the 3D rendering of confocal stacks show the ramified processes of PVM/Ms interfacing with the endothelial tube. Capillaries are labeled with antibody for collagen IV. (E) PVM/Ms contain melanin pigment granules (arrow). (F) Triple labeling for F4/80 (green), GSTα4 (blue), and capillaries (red) in whole-mount SV shows that PVM/Ms express GSTα4.

G Double-labeling for F4/80 (green) and GST (red) in the whole-mount SV shows that PVM/Ms express GST. (H) Double-labeling for F4/80 (green) and Kir4.1 (red) in the whole-mount SV shows that PVM/Ms express Kir4.1. (I) mRNA for Gpf480, Gst, and Kir4.1 was detected in isolated and purified PVM/Ms by RT-PCR analysis. (J and K) Primary cultured PVM/Ms are triple-labeled for GST (red) or GSTα4 (red), F4/80 (green), and nuclei (blue). (L) Primary cultured PVM/Ms are double-labeled for F4/80 (green) and Kir4.1. (M) mRNA for Gpf480, Gst, Gstα4, and Kir4.1 was found in primary cultured PVM/Ms by RT-PCR analysis.
PVM/Ms \((n = 5, P < 0.05)\), indicating that PVM/Ms strengthen the integrity of the endothelial barrier (Fig. 2F).

Transgenic Ablation of PVM/Ms Results in Significant Leakage from Vessels and in Hearing Loss. To validate the in vitro results, we performed an in vivo study in mice in which a transgene encoding a DT receptor was used for transient depletion of PVM/Ms. The mice were assigned randomly to receive DT or control (saline) injections, with an equal volume of saline administered to the control mice. A 5-d regimen of i.v. injections of DT caused a substantial reduction in the number of PVM/Ms \((n = 10; P < 0.01)\) (Fig. 3A and B). The PVM/M-ablated mice showed a significant drop in EP \((B6.FVB-Tg + saline, n = 4; B6.FVB-Tg + DT, n = 6, P < 0.01)\) (Fig. 3C, Right) as well as hearing loss. The hearing loss was not seen in either of the control groups \((C57BL/6J + saline, n = 10; B6.FVB-Tg + saline, n = 10)\) or in DT-treated mice not expressing the receptor \((nC57BL/6J + DT, n = 10; P < 0.05 at 4–16 kHz; P < 0.01 at 24 kHz and 32 kHz)\) (Fig. 3C, Left). Consistent with the results observed in our in vitro model, we found that ablation of PVM/Ms results in marked vascular leakage. Immediately following the 5-d DT treatment, permeability assays using a range of low- and high-molecular-mass fluorescent tracers, including Evans blue, cadaverine Alexa Fluor-555, BSA-Alexa Fluor-555, and IgG-Alexa 568, were found to accumulate in the cochlear lateral wall tissue of PVM/M-ablated animals (Fig. 3D, Right), but not in control animals (Fig. 3D). Immunohistochemical staining in combination with confocal microscopy further demonstrated extravasation of various fluorescent tracers, including cadaverine Alexa Fluor-555 (Fig. 3E, Lower), BSA-Alexa Fluor-555 (Fig. 3F, Lower), and IgG Alexa 568 (Fig. 3G, Lower) in the PVM/M-ablated mice but not in the control mice (Fig. 3 E–G, Upper). The capillaries displayed significant leakiness in the absence of PVM/Ms \((n = 5; P < 0.05)\) (Fig. 3H). The in vivo results corroborate in vitro results showing that absence of PVM/Ms weakens the endothelial barrier. This finding also confirms previous findings that disruption of the intrastrial fluid–blood barrier results in significant hearing loss \((7, 10)\).

PVMs Control Barrier Permeability by Affecting Global Expression of Tight Junction-Associated Proteins. The permeability properties of the intrastrial fluid–blood barrier are largely a function of the tightness of the intercellular junction. The major tight junction-associated proteins in the intrastrial fluid–blood barrier are occludin, claudins, zonula occludens, and adherens-junction proteins \((22)\). Several tight- and adherens-junction proteins, including ZO-1, occludin, and vascular endothelial cadherin (ve-cadherin), have been found in the intrastrial fluid–blood barrier \((23, 24)\). In the in vitro models, mRNA levels for zon-1, occludin, and ve-cadherin, assessed with quantitative RT-PCR (qRT-PCR), were decreased dramatically in the absence of PVM/Ms \((n = 3; P < 0.05)\) (Fig. 4A). Concurrent with decreased mRNA expression, protein levels for ZO-1, occludin, and ve-cadherin, analyzed by in-cell Western blotting, also showed a marked decrease \((n = 5; P < 0.05)\) (Fig. 4B and C). Furthermore, immunohistochemical examination by confocal microscopy clearly showed a less dense distribution of tight- and adherens-junction proteins between ECs in the absence of PVM/Ms (Fig. 4D). The data indicate that PVM/Ms have broad effects on expression of junction proteins, which link directly to endothelial barrier permeability.
The impact of PVM/Ms on the expression of junction proteins was validated further with qRT-PCR analysis and immunofluorescent analysis on isolated strial capillaries from control and PVM/M-ablated animals. A previously established sandwich-dissociation method (25) was used to isolate and separate capillaries (Fig. 4E) and tight junction-associated proteins from adjoining marginal and basal cell layers with minimal contamination. Tight- and adherens-junction expression was assessed in the control and PVM/M-ablated animal groups. Consistent with the results of the in vitro study, mRNA for ZO-1, occludin, and v-cadherin, determined by qRT-PCR, was reduced significantly in the absence of PVM/Ms (Fig. 4F). Isolated capillaries from the control mice displayed strong patches of immunohistochemical staining for in situ-expressed ZO-1, occludin, and ve-cadherin protein (Fig. 4 G–I, Left); the staining was decreased dramatically in the PVM-depleted cochlear capillaries of PVM/M-ablated animals (Fig. 4 G–I, Right). Ablation of PVM/Ms significantly reduced expression of ZO-1, occludin, and ve-cadherin (n = 3; P < 0.05) (Fig. 4 J–L). The in vivo result further validates our hypothesis that PVM/Ms control barrier integrity by affecting the expression of tight-junction-associated proteins.

**PVMs Exert an Effect on Barrier Integrity Through Focal Secretion of PEDF.** Next we examined the mechanism by which PVM/Ms affect expression of junction proteins. PEDF is a 50-kDa glycoprotein previously found expressed in the rat inner ear, including a purpose for siRNA targeting PVM/Ms. qRT-PCR analysis showed a 60% down-regulation of Pedf gene expression in vivo by siRNA (Fig. 5L). Consistent with the down-regulation of ZO-1, occludin, and ve-cadherin seen in vitro, down-regulation of Pedf gene expression by siRNA resulted in dramatically decreased protein expression in capillaries (Fig. 5 L–N, Left) as compared with a vector control group (Fig. 5 M–O, Center). Application of PEDF effectively ameliorated siRNA-induced suppression of ZO-1, occludin, and ve-cadherin protein expression (Fig. 5 M–O, Right), whereas inhibition of PEDF significantly reduced the expression of tight- and adherens-junction proteins (Fig. 5 P–R, experiments in triplicate, P < 0.05). The results implicate PEDF signaling between PVM/Ms and ECs as an important mediator of the effect PVM/Ms have on expression of tight- and adherens-junction proteins.

**Discussion**

Our experiments show that perivascular-resident cells, identified here as melanocyte-like macrophages, have an important role in regulating the integrity of the intrastrial fluid–blood barrier and for maintaining normal hearing thresholds. In vitro, the absence of PVM/Ms increases the permeability of an EC monolayer. The results are confirmed in vivo, where ablation of PVM/Ms causes increased capillary leakage with an accompanying substantial drop in EP and hearing loss. These effects are mediated through PEDF, which directly affects the expression of tight junction-associated proteins such as occludin, ZO-1, and ve-cadherin.

The intrastrial fluid–blood barrier is one of the tightest blood-tissue barriers known in mammals. It is situated between blood flow and the intrastrial region of the SV. This region is critical for maintaining inner ear homeostasis, especially for maintaining the EP, an essential driving force for hearing function. At the
vascular-resident macrophages are positive for melanocyte marker proteins, con-
perring pericytes. In this study, we found that the perivascular-resident
microvessels and structurally were intertwined with ECs and
and CD11b. The macrophages were closely associated with
several macrophage surface molecules, including F4/80, CD68,
vascular-resident macrophages, because they were positive for
SV of normal adult cochlea. The cells were identi-
the SV comprises three cell layers (marginal, intermediate, and
membrane (2). Like other blood
lining cochlear microvessels and an associated basement mem-
absence of fenestrations (11). Results from this study highlight
blood barrier is formed by tight junctions between ECs with the
morphological complexity of the barrier in the SV, which
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PVM/M control of barrier integrity is mediated by PEDF. (A) Cultured
PVM/Ms express PEDF (Upper), whereas ECs express its receptor PEDFR
(Lower). (B) PEDF production in a 4-d PVM/M culture medium. (C) Triple-la-
beled whole-mount SV shows PEDF (Top) strongly expressed in PVM/Ms
(Middle). (Bottom) Merged image displaying capillary staining with antibody
for IgG. (D) (Top) Isolated capillaries labeled with antibody for collagen IV
(Middle) PEDF is expressed in the capillary wall. (Bottom) Merged image. (E) In
vitro siRNA-transfected cells (green) double labeled for PVM/M marker
protein F4/80 (red) and nuclei (DAPI, blue). (F) qRT-PCR analysis shows down-
regulated mRNA expression of Pedf in the transfected PVM/Ms. (G) qRT-PCR
analysis shows levels of mRNA for zo-1, occludin, and ve-cadherin in siRNA-
transfected, control, and PEDF-treated EC monolayers. (H–J) In-cell Western
blot protein analysis for Zo-1, occludin, and ve-cadherin in siRNA-transfected,
control, and PEDF treated EC monolayers. (K) The confocal and DIC image
shows siRNA-transfected cells (green) in the SV in vivo. (L) qRT-PCR shows
down-regulated mRNA expression for Pedf in PVM/Ms in vivo. (M–O) Ex-
pression of the proteins in isolated capillaries of siRNA-transfected (Left),
vector control (Center), and PEDF-treated PVM/M-depleted animals (Right).
(P–R) Quantitative analysis of the proteins. *P < 0.05; **P < 0.01.

cellular level, the intrastrial fluid–blood barrier comprises ECs
lining cochlear microvessels and an associated basement mem-
brane (2). Like other blood–tissue barriers, the intrastrial fluid–
blood barrier is formed by tight junctions between ECs with the
absence of fenestrations (11). Results from this study highlight
the morphological complexity of the barrier in the SV, which
includes surrounding basal lamina as well as a second line of
support in PVM/Ms.

In a previous study, a large population of perivascular cells was
found in the vicinity of the intrastrial fluid–blood barrier in the
SV of normal adult cochlea. The cells were identified as peri-
vascular-resident macrophages, because they were positive for
several macrophage surface molecules, including F4/80, CD68,
and CD11b. The macrophages were closely associated with
microvessels and structurally were intertwined with ECs and
pericytes. In this study, we found that the perivascular-resident
macrophages are positive for melanocyte marker proteins, con-
tain significant amounts of melanin, and express Kir 4.1, the fi-
duciary marker of intermediate cells (28). In the standard view,
the SV comprises three cell layers (marginal, intermediate, and
basal cell) and a rich capillary network. Earlier work had dis-
tinguished two subclasses of intermediate cells (29). We suspect
the dark, melanocyte subclass of these intermediate cells is the
equivalent of PVM/Ms. As a matter of fact, Conlee et al. (30)
provide evidence for a distinction between melanocytes and in-
termediate cells in cats. Likewise, our studies show that PVM/Ms
are a hybrid cell type, including characteristics of both macro-
phage and melanocyte phenotypes. Given their high density on
capillary walls, an understanding of their function in the intras-
trial fluid–blood barrier is critically important.

We hypothesize that the integrity of the intrastrial fluid–blood
barrier is dependent on the structural association between PVM/
Ms and ECs, with the process endings integral to the re-
strictiveness of the barrier. Although the in vitro model provides
direct measurement of the interaction between ECs and PVM/
Ms, some features of the in situ intrastrial fluid–blood barrier
may be missing. Therefore we validated our findings with a transgenic (Tg) mouse model in which a DT receptor was
expressed under the control of a human integrin αM promoter
(CD11b). This mouse strain provided a system for transient
depletion of PVM/Ms without toxic side effects (17, 18). In ad-
dition to leakage, PVM/M-ablated mice had hearing loss, dem-
onstrating that a functional intrastrial fluid–blood barrier is
required for normal hearing. When DT was administered to
control mice, no side effects or toxicity was found. In mice not
expressing the receptor, DT injection had no effect on hearing
thresholds. However, in the DT receptor-fused mice, we found
PVM/M depletion caused significant leakage in capillaries. In

Fig. 5. PVM/M control of barrier integrity is mediated by PEDF. (A) Cultured
PVM/Ms express PEDF (Upper), whereas ECs express its receptor PEDFR
(Lower). (B) PEDF production in a 4-d PVM/M culture medium. (C) Triple-la-
beled whole-mount SV shows PEDF (Top) strongly expressed in PVM/Ms
(Middle). (Bottom) Merged image displaying capillary staining with antibody
for IgG. (D) (Top) Isolated capillaries labeled with antibody for collagen IV
(Middle) PEDF is expressed in the capillary wall. (Bottom) Merged image. (E) In
vitro siRNA-transfected cells (green) double labeled for PVM/M marker
protein F4/80 (red) and nuclei (DAPI, blue). (F) qRT-PCR analysis shows down-
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blot protein analysis for Zo-1, occludin, and ve-cadherin in siRNA-transfected,
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shows siRNA-transfected cells (green) in the SV in vivo. (L) qRT-PCR shows
down-regulated mRNA expression for Pedf in PVM/Ms in vivo. (M–O) Ex-
pression of the proteins in isolated capillaries of siRNA-transfected (Left),
vector control (Center), and PEDF-treated PVM/M-depleted animals (Right).
(P–R) Quantitative analysis of the proteins. *P < 0.05; **P < 0.01.

Fig. 6. Working model of the PEDF/PEDFR signaling pathway regulating the
expression of junction proteins. Capillary ECs are coupled by tight and adherens
junctions that limit paracellular transport between adjacent cells. Circulating
large molecules are extravasated from the capillary when PVM/Ms are absent.
PVM/Ms control vascular permeability by affecting the expression of tight- and
adherens-junction proteins through a PEDF/PEDFR signaling pathway.
addition to leakage, PVM/M-ablated mice showed a substantial drop in EP accompanied by significant hearing loss. The EP, a voltage gradient essential for hearing, requires a functional capillary network. Hearing loss with PVM/M depletion may be the direct result of low EP from either breakdown of the barrier or loss of melanocytes. Melanocytes are essential for EP generation (31, 32), whereas disruption of the barrier results in an intrastriatal electric shunt (7).

We next investigated possible mechanisms by which PVM/Ms effect changes in vascular permeability. Two transport pathways between the blood compartment and irrigated tissue are known (20): transcellular and paracellular passage. Transcellular passage through ECs requires cell fenestration or a complex system of trafficking vesicles; whereas the paracellular pathway depends on coordinated opening and closing of EC junctions. The permeability of the endothelium is largely a function of the tightness of the intercellular junctions. In an earlier mass spectrometry study, strial capillaries were found rich in tight-junction and cell-adhesion proteins (24). The major tight junction-associated proteins in the strial capillaries are occludin, claudins, ZO-1, and adherens-junction proteins (23, 24). The present study found that tight-junction and adhesion proteins were down-regulated substantially in the absence of PVM/Ms. The down-regulation was associated with PEDF production. Application of PEDF to the tissue dramatically ameliorated the effect of PVM/M absence on leakage and attenuated the deregulation of junction proteins induced by absence of PVM/Ms (illustrated schematically in Fig. 6).

PEDF is a 50-kDa secreted glycoprotein of the noninhibitory serpin family that exerts diverse physiological effects, including angiogenic and antivascular permeability, antitumor, and neurotrophic effects (21). Studies have shown that PEDF binding to its receptor counteracts VEGF-induced vascular permeability (33, 34). Other studies point to PEDF suppression of VEGF-induced vascular permeability by targeting the PI3K/Akt signaling pathway (34) or phosphorylating adherens-junction proteins by a γ-secretase–mediated pathway (33). Our findings demonstrate that secretion of PEDF by PVM/Ms has direct and broad effects on the expression of several tight junction-associated proteins including occludin, ZO-1, and ve-cadherin.

In conclusion, this study has shown that PVM/Ms, a hybrid phenotype with macrophage and melanocyte characteristics, are important for hearing and that they control the integrity of the intrastriatal fluid–blood barrier in the SV by affecting the expression of tight- and adherens-junction proteins. We established the important role of PVM/Ms in stabilizing the intrastriatal fluid–blood barrier and identified PEDF as an essential signaling molecule.

**Materials and Methods**

Male C57BL/6J and B6.FVB-Tg (ITGAM-DTR/EGFP) 3AluN1 mice that encode a DT receptor fused with GFP under the control of a human integrin αM (ITGAM) promoter (CD11b) were used in this study. The Tg mice provide a DT-inducible system for transient depletion of macrophages. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University (IACUC approval number: B11265). All reagents and detailed experimental procedures used in this study, including primary PVM/M and EC cultures, isolation of strial capillaries, measurement of endothelial barrier permeability in vitro and in vivo, cell Western blot analysis, immunohistochemistry, confocal microscopy and immunofluorescence analysis, ELISA, qRT-PCR, transfection with siRNA, PVM/M counts, drug treatment, measurements of EP and auditory brainstem response, electron microscopy, and statistical analyses are described in **Materials and Methods**.

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**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: 060000) 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-mm³) with ophthalmonic 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% FBS, and 0.5% gentamicin/amphotericin. 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% CO₂ for 10 min at 4°, 1 mL cold isolation buffer was added for 1 min, and then removed carefully. The supernatant was discarded, harvested, and used to determine the passage number, density seeded and grown on the inserts for 5 d. Isolation buffer (1 mL) was added and gently pipetted in the same concentration at day 5 (5 μL/mL, 200 μL per well) were seeded 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 μL/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) paraformaldehyde 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% parafomaldehyde (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 immunoblotted with a so-
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% (vol/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.
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) 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 (Ct) method (ΔΔCt 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 \((F_T - F_{0T})/(F_C - F_{0C}) \times 100\%\), where \(F_T\) is the average fluorescence intensity in treated groups, \(F_C\) is the average fluorescence intensity in control groups, and \(F_{0T}\) and \(F_{0C}\) are 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 x 10³ 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). Mice 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 Ct method (ΔΔCt 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 20x 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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interleukin-1 beta-induced vascular permeability and angiogenesis in retinal endothelial cells. Vascul Pharmacol 52:84–94.

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 end-feet (dark blue arrow).

Table S1. Primary antibodies employed

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