Published in final edited form as:

Nat Protoc. 2013 April ; 8(4): 709–720. doi:10.1038/nprot.2013.033.

Isolation and culture of endothelial cells, pericytes and perivascular resident macrophage-like melanocytes from the young mouse ear

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Abstract

This protocol describes a growth medium–based approach for obtaining cochlear endothelial cells (ECs), pericytes (PCs) and perivascular resident macrophage-like melanocytes (PVM/Ms) from the stria vascularis of mice aged between P10 and P15 (P, postnatal day). The procedure does not involve mechanical or enzymatic digestion of the sample tissue. Explants of stria vascularis, ‘mini-chips’, are selectively cultured in growth medium, and primary cell lines are obtained in 7–10 d. The method is simple and reliable, and it provides high-quality ECs, PVM/Ms and PCs with a purity >90% after two passages. This protocol is suitable for producing primary culture cells from organs and tissues of small volume and high anatomical complexity, such as the inner ear capillaries. The highly purified primary cell lines enable cell culture–based *in vitro* modeling of cell-cell interactions, barrier control function and drug action.

INTRODUCTION

The cochlear blood-labyrinth barrier tightly regulates the cochlear microenvironment for auditory function^{1–4}. PCs, PVM/Ms and ECs are crucial components of the blood-labyrinth barrier and are essential for maintaining blood-labyrinth barrier integrity⁵. However, investigation of these cell types has been hindered by the lack of a method for isolating and culturing them.

Over the past few decades, *in vitro* cell-based models, widely used in studies of the blood-brain barrier and blood-retina barrier, have been powerful tools for studying cell-cell interactions^{6–8}. Several different methods for isolating and culturing cells from the adrenal

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AUTHOR CONTRIBUTIONS W.Z. and L.N. created the initial protocol. L.N. refined the protocol. A.H. and M.Z. performed the SEM imaging. A.K. supervised the flow cytometry. X.S., A.F. and M.A. supervised the project. X.S. wrote the manuscript. All the authors discussed the results, procedures and commented on the manuscript at different stages.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

gland, umbilical vein, lung, skeletal muscle, brain and kidney have been reported⁹⁻¹⁹. The reported methods, however, are not suitable for isolating blood-labyrinth barrier cells. The small volume and anatomical complexity of capillaries in the inner ear presents unique challenges, and extraction and isolation methods designed for larger tissue volumes have not been practical for culturing cells from the stria barrier. Our limited knowledge of the cellular and functional components of the blood-labyrinth barrier is partly due to the lack of primary ECs, PCs and PVM/Ms from the mouse inner ear to experiment on.

In this study, we describe a novel growth medium-based method for obtaining EC, PC or PVM/M primary cultures from tiny explants (mini-chips) of stria vascularis tissue, first described in ref. 5. Tissue is harvested from 10- to 15-d-old mice. In mice of this age, the stria vascularis is fully formed and separated from the spiral ligament and the cells are still highly proliferative. The tearing of the tissue into mini-chips provides for sufficient penetration of the growth culture medium. The mixed population of stria cell types is grown in specific culture medium to selectively support the growth of each phenotype. The unwanted phenotypes do not survive passaging. The harvesting process takes less than 2 h and does not require additional equipment or special enzyme treatment. Primary cell types are generated within 7–10 d. Purities of >90% are obtained for the cultured primary ECs, PCs and PVM/Ms after two passages (~3 weeks). The protocol is simple and provides consistent results. The overall procedure and sequence of steps for isolation and culture of ECs, PCs and PVM/Ms from the young mouse ear is given in Figures 1 and 2.

Experimental design

The auditory bulla is dissected under sterile conditions from mice aged P10–P15 and placed in cold artificial perilymph solution (Fig. 1). Collection of six stria vascularis explants, yielding enough cells for propagation of each cell line, can be completed in less than 1 h (Fig. 1a). Mini-chips of stria vascularis explants are produced from whole-mounted tissue, and the procedure for seeding the fragments is completed in less than 1 h (Fig. 1c–g). The mini-chips of the stria vascularis, containing ECs, PCs and PVM/Ms, are then selectively cultured in one of the three specific growth media depending on whether ECs, PCs or PVM/Ms are required. Multiple cell clusters should form around the explanted stria vascularis chips by day 1 or 2; these then expand and spread over the surface of the Petri dish by day 6. In general, primary cell lines are obtained in 7–10 d (Fig. 2a–c). In all, 12×10^4 ECs, 5×10^4 PCs and 9×10^4 PVM/Ms are generated in the initial (P1) stage. ECs, PCs and PVM/Ms are passaged and further selected with specific growth medium.

By passage 3, $2\text{--}3 \times 10^6$ ECs, $1\text{--}2 \times 10^6$ PCs and $1.5\text{--}2.5 \times 10^6$ PVM/Ms should have been obtained. The cell phenotype and purity can then be assessed via immunohistochemistry, reverse transcriptase PCR (RT-PCR) and flow cytometry.

Applications of the method

ECs, PCs and tissue-resident macrophages have shown organ-specific differences in experiments in other tissues^{20,21}. Primary cells generated from the ear enable us to obtain information on the organ-specific characteristics of the cochlear blood-tissue barrier. In particular, the primary cell lines enable direct study of intercellular interactions between ECs, PCs and PVM/Ms in coculture models, which is relevant for a better understanding of transport processes, including drug delivery. The models provide new insight into the mechanisms of the cochlear blood-tissue barrier, particularly for the specific contribution of component cells to blood-labyrinth barrier integrity. These newly developed primary cell lines have enabled the identification of the role of PVM/Ms in controlling the integrity of the intrastrial fluid-blood barrier⁵.

Cell lines gradually lose their phenotype with passaging. The method provides a ready source of barrier cell types. Moreover, as confluent monolayers of cell lines can be shipped to other laboratories, experiments with the *in vitro* model using standardized protocols on the same cell stock can be repeated for robust testing of hypotheses.

Comparison with other methods, including our previously published method

To our knowledge, this is the first protocol that describes a method for culturing the three primary cell types of the blood-labyrinth barrier from the mouse ear (ECs, PCs and PVM/Ms). This method is qualitatively different from the methods previously published for obtaining comparable cell lines. Typically, the cell lines are cultured from brain, umbilical vein, lung or kidney tissues in mouse, rat, bovine and human models, and initial cell number has not been a limiting factor for these methods. The blood-labyrinth barrier in the stria vascularis consists of less than 5,000 cells in one ear, and methods that rely on abundant initial stock are not practical. The current protocol is a modified version of the protocol we published earlier⁵, but is as used in ref. 22.

In our initial protocol, we used mice that were a couple of days younger (P7–P10). We encountered difficulties in working with mice at this age, as, within the range of genetic differences, the stria vascularis was not consistently fully formed and separated from the spiral ligament (according to Iwagaki *et al.*²³, external wall vessels are a single-layer capillary network at birth, and this single layer subsequently divides into two layers, constituting the microvessels of the stria vascularis and spiral ligament between days 5 and 8 in mice). In addition, tissue and bone at this age are very soft and fragile, and it is difficult to obtain good separation. Frequently, tissues were mechanically crushed, with major cell damage. In the current version of the protocol, mice aged P10–P15 are used, which presents less of a surgical challenge, and the rigidity of the stria vascularis lends itself to easy detachment from the spiral ligament. Most notably, the cells are still highly proliferative.

Previously, pieces of the stria vascularis were cut to ~1 mm³ in size. Although the first passage of the primary cell line could be generated in a 10-d frame, cell yield was much lower. The slower growth is attributed to the restricted access that larger-sized explants have to the nutrients in the growth medium. The blood-labyrinth barrier in the stria vascularis²⁴ is sandwiched between epithelial marginal cells and the basal cells interconnected by tight junctions. Blood-barrier component cells may be sensitive to nutrient level and show less growth under starvation conditions. In the current protocol, the stria vascularis is torn into pieces the size of little seeds (0.15–0.20 mm³) and uniformly bathed in their growth medium. This procedure reliably produces a large population of ECs, PCs and PVM/Ms.

We established the previous protocol to answer a more restricted question—whether PVM/Ms affect blood-labyrinth barrier integrity—and for this purpose, we needed only ECs and PVM/Ms from the stria vascularis explants. With the current chip method, a cell line of the third major barrier cell type (PCs) is produced. A major concern in PC cell lines is EC contamination^{18,25,26}. In our protocol, the culture medium for PC growth contains pigment epithelium-derived growth factor (PEDF) at a concentration of 100 nM. This growth factor promotes PC proliferation and reduces EC contamination by suppressing EC growth^{27,28}.

Overall, this method of producing high-quality ECs, PCs and PVM/Ms is simple and reliable. This method is especially suitable for obtaining these cell types from organs and tissues of small volume and high anatomical complexity, such as the capillaries of the inner ear. Although cells can be obtained by experimenters with very little training, results improve over a 2–3-month time frame.

MATERIALS

REAGENTS

Surgery and dissection reagents

- Mice aged 10–15 d (C57BL/6J; either sex can be used) **! CAUTION** Experiments involving live rodents must conform to all relevant governmental and institutional regulations.
- Ketamine hydrochloride, injectable, 100 mg ml⁻¹ (JHP Pharmaceuticals, cat. no. 074666)
- Xylazine, sterile solution, 100 mg ml⁻¹ (Lloyd, cat. no. LPFL04821)
- Euthanasia solution, 100 ml (Virbac Animal Health, cat. no. 710101)
- Ethanol, 70% (vol/vol; Sigma, cat. no. 459844)
- Sodium chloride, 500 g (NaCl; Fisher Scientific, cat. no. S271-500)
- Potassium chloride, 500 g (KCl; Fisher Scientific, cat. no. P217-500)
- Calcium chloride hexahydrate, 1 kg (CaCl₂·6H₂O; Sigma, cat. no. 442909)
- Magnesium chloride hexahydrate, 500 g (MgCl₂·6H₂O; Fisher Scientific, cat. no. M33-500)
- Sodium phosphate monobasic, 500 g (NaH₂PO₄·H₂O; Fisher Scientific, cat. no. S369-500)
- HEPES, 500 g (Fisher Scientific, cat. no. BP310-500)
- D-(+)-Glucose, 1 kg (Sigma, cat. no. G7528)
- Lidocaine HCl

Cell culture reagents

- DMEM, 500 ml (Life Technologies, cat. no. 11885-084)
- CS-C medium without serum for endothelial cell lines, 100 ml (Sigma, cat. no. C1556)
- Medium 254CF, 500 ml (Life Technologies, cat. no. M-254CF-500)
- FBS, 500 ml (Sigma, cat. no. F2442)
- Endothelial cell growth factor, 100× (ECGF; Sigma, cat. no. E9640)
- Pigment epithelium-derived factor, 20 µg (PEDF human; Sigma, cat. no. SRP4988)
- Human melanocyte growth supplement, 100× (HMGS; Life Technologies, cat. no. S-002-5)
- Penicillin-streptomycin solution, 100 ml (10,000 units of penicillin and 10 mg of streptomycin per ml; Sigma, cat. no. P4333)
- Gentamicin/amphotericin B, 500× (Life Technologies, cat. no. 50-0640)

Cell purity assessment reagents

- Trypsin-EDTA solution (0.25%, wt/vol), 500 ml (Sigma, cat. no. T4049)

- Lectin from *Bandeiraea simplicifolia*, 1 mg ml⁻¹ (*Griffonia simplicifolia*; Isolectin B₄ (BSI-B₄); FITC conjugate; Sigma, cat. no. L2895) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- Rat monoclonal antibody (APB5) to platelet-derived growth factor receptor-β (PDGFR-β), 0.2 mg ml⁻¹ (phycoerythrin; Abcam, cat. no. ab93534) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- Anti-F4/80 antibody (CI:A3-1), 0.1 mg ml⁻¹ (phycoerythrin; Abcam, cat. no. ab105156) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- Rat IgG phycoerythrin— isotype control, 0.1 mg ml⁻¹ (Abcam, cat. no. ab37368) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- D-(+)-Galactose, 25 g (Sigma, cat. no. G0750)

Immunocytochemical reagents

- Paraformaldehyde (32% (vol/vol); PFA; Electron Microscopy Sciences, cat. no. 15714-S) ! **CAUTION** PFA is a carcinogenic reagent. Wear gloves and eye protection and avoid inhalation of the reagent.
- PBS (pH 7.2), 500 ml (Life Technologies, cat. no. 20012-027)
- Triton X-100, 1 liter (Sigma, cat. no. T9284)
- BSA, 100 g (Fisher Scientific, cat. no. BP1600-100)
- Donkey serum, 25 ml (Abcam, cat. no. ab7475)
- Goat serum, 10 ml (Sigma, cat. no. G9023)
- von Willebrand factor—specific antibody, 500 μl (vWF; Abcam, cat. no. ab11713)
- Isolectin GS-IB4 from *Griffonia simplicifolia*, 1 mg ml⁻¹; Alexa Fluor 568 conjugate (Life Technologies, cat. no. I21412)
- Alexa Fluor 488 donkey anti-sheep IgG (H + L), 2 mg ml⁻¹ (Life Technologies, cat. no. A-11015) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- Anti-mouse F4/80 antigen, purified, 0.5 mg ml⁻¹ (eBioscience, cat. no. 14-4801-85)
- Alexa Fluor 568 goat anti-rat IgG (H + L), 2 mg ml⁻¹ (Life Technologies, cat. no. A-11077) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- Anti-MiTF antibody, 0.6 mg ml⁻¹ (Abcam, cat. no. ab20663)
- Anti-desmin antibody [Y266], 0.018 mg ml⁻¹ (Abcam, cat. no. ab32362)
- Alexa Fluor 568 goat anti-rabbit IgG (H + L), 2 mg ml⁻¹ (Life Technologies, cat. no. A-11011) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- Anti-PDGF receptor-β antibody [Y92], 0.24 mg ml⁻¹ (Abcam, cat. no. ab32570)

- Alexa Fluor 488 goat anti-mouse IgG (H + L), 2 mg ml⁻¹ (Life Technologies, cat. no. A-11001) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- ZO-1 rabbit pAb, 0.25 mg ml⁻¹ (Life Technologies, cat. no. 61-7300)
- NucBlue fixed cell stain, 1 kit (DAPI special formulation; Life Technologies, cat. no. R37606)
- Alexa Fluor 488 phalloidin (300 units; Life Technologies, cat. no. A12379) ■ **CAUTION** This reagent is toxic. Avoid inhalation, ingestion and skin contact.

RT-PCR reagents

- RNeasy micro kit (50; Qiagen Sciences, cat. no. 74004)
- RETROscript kit reverse transcription for RT-PCR (Life Technologies, cat. no. AM1710)
- SuperTaq polymerase, 250 U (Life Technologies, cat. no. AM2052)
- GeneRuler 100-bp DNA ladder, 50 µg (Thermo Fisher Scientific, cat. no. SM0243)
- 6× DNA loading dye, 1 ml (Thermo Fisher Scientific, cat. no. R0611)
- Tris-borate-EDTA, 4 liters, 1× solution (TBE; Fisher Scientific, cat. no. BP2430-4)
- UltraPure agarose-1000, 100 g (Life Technologies, cat. no. 10975-035)
- SYBR Safe DNA gel stain, 400 µl (Life Technologies, cat. no. S33102) ▲ **CRITICAL** This reagent is light sensitive. Avoid exposure to light by wrapping the reagent tube with tinfoil.
- PCR primers (synthesized by Integrated DNA Technologies):
 - GAPDH forward: 5'-ATGTGTCCGTCGTGGATCTGA-3'
 - GAPDH reverse: 5'-AGACAACCTGGTCCTCAGTGT-3'
 - NG2 forward: 5'-CAGGCCGGTCGGGTGACCTA-3'
 - NG2 reverse: 5'-GGGCCACGTGGAAGACACGG-3'
 - PDGFRβ forward: 5'-ACCTGCAGAGACCTCAAAAGTAGGT-3'
 - PDGFRβ reverse: 5'-ACCACGGTGACCTCCTGCGA-3'
 - Desmin forward: 5'-AGCCAGCGCGTGTCTCCTA-3'
 - Desmin reverse: 5'-AGCGTCGGCCAGGAGAAAGT-3'
 - CD34 forward: 5'-GGGAGCCACCAGAGCTATTC-3'
 - CD34 reverse: 5'-CACCACATGTTGTCTTGCTGA-3'
 - vWF forward: 5'-TGTTTCATCAAATGGTGGGCAGC-3'
 - vWF reverse: 5'-ACAGACGCCATCTCCAGATTCA-3'
 - Glut1 forward: 5'-GCTGTGCTTATGGGCTTCTC-3'
 - Glut1 reverse: 5'-AGAGGCCACAAGTCTGCATT-3'
 - GSTα4 forward: 5'-GCTGCGGCTGGAGTGGAGTTTG-3'
 - GSTα4 reverse: 5'-TGCCCAACTGAGCTGGTTGCC-3'

- F4/80 forward: 5'-TGCATCTAGCAATGGACAGC-3'
- F4/80 reverse: 5'-GCCTTCTGGATCCATTTGAA-3'
- GST forward: 5'-CGCCACCAAATATGACCTCT-3'
- GST reverse: 5'-CCTGTTGCCCAAGGTAGT-3'

Scanning electron microscopy (SEM) reagents

- Glutaraldehyde, 25% (wt/vol) aqueous solution, EM grade (Ted Pella, cat. no. 18426) **! CAUTION** This reagent is corrosive. Wear gloves and eye protection, and avoid inhalation of the reagent.
- PBS, tablets (Sigma-Aldrich, cat. no. P4417-50TAB)
- Osmium tetroxide, 4% (wt/vol) aqueous solution (Electron Microscopy Sciences, cat. no. 19190) **! CAUTION** This reagent is highly corrosive. Wear gloves and eye protection, and avoid inhalation of the reagent. Work in a fume hood.
- Ethyl alcohol, absolute (200-proof; Electron Microscopy Sciences, cat. no. 15055)
- Poly-L-lysine solution, (0.1% (wt/vol); Sigma-Aldrich, cat. no. P8920-100ML)
- PFA, 16% (vol/vol) formaldehyde solution, EM grade (Electron Microscopy Sciences, cat. no. 15710) **! CAUTION** This reagent is corrosive. Wear gloves and eye protection, and avoid inhalation of the reagent.
- Sodium cacodylate buffer, 0.2M (Electron Microscopy Sciences, cat. no. 11650)

Cell storage reagents

- DMSO, 500 ml (EMD Chemicals, cat. no. MX1458-6)
- Trypan blue stain (0.4% (wt/vol); Life Technologies, cat. no. 15250-061)

EQUIPMENT

Surgery, dissection and cell culture equipment

- Biosafety cabinet (hood) suitable for cell culture and equipped with UV light for decontamination
- Water bath with temperature control (Fisher Scientific, model no. Isotemp 2100)
- Sterilimatic sterilizer (Market Forge, model no. STM-EL)
- Cell culture incubator with both temperature and gas composition controls (Thermo Scientific, model no. 3110)
- Stereomicroscope (Olympus, model no. SZ61) with swan-neck fiber optic illumination (Olympus, model no. 3100)
- Polypropylene conical tubes, 50 ml (BD Biosciences, cat. no. 352070)
- Filtration units for solutions, 0.22 μ m (Fisher Scientific, cat. no. 09-719A)
- Sterile Petri dishes, 35 mm (BD Biosciences, cat. no. 351008)
- Tissue culture dishes, collagen I coated, 35 mm (BD Biosciences, cat. no. 356456)
- Tissue culture dishes, collagen I coated, 60 mm (BD Biosciences, cat. no. 356401)
- Graefe forceps, 0.8-mm straight tips (Fine Science Tools, cat. no. 11050-10)

- Dumont forceps, standard tip, 0.10 mm × 0.06 mm, Dumoxel, 11 cm (Fine Science Tools, cat. no. 11251-30)
- Serological pipettes, 10 ml (BD Biosciences, cat. no. 357551)

Cell purification equipment

- MPR centrifuge, 1 liter (Thermo Scientific, model no. 120)
- Inverted microscope with phase contrast (Nikon, model no. TS 100) and digital camera (Nikon, model no. Coolpix 990)
- Hemocytometer set (Hausser Scientific, model no. 1483)
- BD Influx cell sorter (BD Biosciences, model Influx)
- Polypropylene round-bottom tubes, 5 ml (BD Biosciences, model no. 352063)

Phenotype validation equipment

- Collagen-coated glass-bottom dish, 35 mm (MatTek, model no. P35GCOL-1.5-10-C)
- Confocal laser microscope (Olympus, model no. FV1000)
- UV-visible spectrophotometer (Thermo Scientific, model NanoDrop 1000)
- Thermal cycler (Bio-Rad Laboratories, model MyCycler)
- Power supply (Bio-Rad Laboratories, model PowerPac HC)
- Horizontal electrophoresis cells (Bio-Rad Laboratories, model Mini-Sub CellGT)
- Bio-Rad universal hood II gel imager (Bio-Rad Laboratories, model Universal Hood II)

SEM equipment

- SEM stubs (self-made)
- Double-sided carbon tape, 12 mm × 20 m (SPI supplies, cat. no. 05082-AB)
- Sapphire disks, 3 mm (Leica Microsystems, cat. no. 16702766)
- Critical point dryer (Tousimis, model no. AutoSamdri 815)
- Sputter coater (Polaron SEM coating system)
- Scanning electron microscope (Hitachi, model no. S-5000)
- Dumont tweezers, biological grade, 0.03 mm × 0.07 mm, Dumoxel (Electron Microscopy Sciences, cat. no. 72864-D)

Cell storage equipment

- Cryogenic vial, 2.0 ml (Corning, cat. no. 430659)
- Nalgene Cryo 1 °C freezing container (Thermo Scientific, cat. no. 5100-0001)
- Forma -86 °C ULT freezer (Thermo Scientific, model no. 993)
- Liquid nitrogen tank (Thermo Scientific)

Cell coculture equipment

- 24-well plates (BD Biosciences, cat. no. 353047)

- Cell culture inserts, 3.0 μm (BD Biosciences, cat. no. 353096)
- CytoVu/SiMPore thin membrane coculture system (SiMPore, cat. no. c300-MP3)

REAGENT SETUP

Anesthetic—For 1 ml of anesthetic solution, combine 0.2 ml of 100 mg ml^{-1} ketamine hydrochloride, 0.2 ml of 20 mg ml^{-1} xylazine and 0.6 ml of bacteriostatic 0.9% (wt/vol) NaCl. The solution should be freshly prepared before use.

Isolation medium (artificial perilymph solution)—To prepare isolation medium, add 125 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl_2 , 1.5 mM MgCl_2 , 0.51 mM NaH_2PO_4 , 10 mM HEPES and 5 mM glucose at pH 7.4, with osmolarity adjusted to 310 mmol kg^{-1} . Filter the solution (0.22- μm filter) and keep it on ice throughout the experiment. The prepared solution can be used for up to 1 week when stored at 4 °C.

EC culture medium—For 100 ml of medium, combine 10 ml of FBS, 1 ml of penicillin-streptomycin solution, 1 ml of ECGF and 88 ml of CS-C medium without serum. Strain the medium through a 0.22- μm filter.

PC culture medium—For 100 ml of medium, combine 10 ml of FBS, 1 ml of penicillin-streptomycin solution and 89 ml of DMEM low glucose. Add PEDF to the solution to a final concentration of 100 nM.

PVM/Ms culture medium—For 100 ml of medium, combine 10 ml of FBS, 200 μl of gentamicin/amphotericin B solution, 1 ml of HMGS and 88.8 ml of medium 254CF. Add CaCl_2 (provided with medium 254CF) to the solution to a final concentration of 0.2 mM. ▲ **CRITICAL** All culture media should be labeled with the storage conditions and expiration date. We recommend using prepared solutions for only up to 1 week when stored at 4 °C.

PBS-BSA solution, 1% (wt/vol)—Dissolve 1 g of BSA in 100 ml of PBS ▲ **CRITICAL** The solution can be stored at 4 °C for several weeks.

Immunofluorescence blocking solution—For 10 ml of a 10% (vol/vol) goat serum PBS-BSA solution, combine 1 ml of goat serum and 9 ml of 1% (wt/vol) PBS-BSA solution. For 10 ml of a 10% (vol/vol) donkey serum PBS-BSA solution, combine 1 ml of donkey serum and 9 ml of 1% (wt/vol) PBS-BSA solution. ▲ **CRITICAL** The solution can be stored at −20 °C for several weeks.

Agarose gel, 1.5% (wt/vol)—Add 0.45 g of agarose powder and 3 μl of SYBR Safe DNA gel stain to 30 ml of 1 × TBE buffer. Carefully heat the mixture until the agarose is completely dissolved. Allow the resulting solution to cool at room temperature (25 °C) for 20 min. Pour the solution immediately into a casting tray. Allow the gel to form at room temperature over a period of at least 30 min. The gels are stable for several weeks at 4 °C when protected from light.

Cryopreservation medium—Supplement the cell complete medium with 10% (vol/vol) DMSO and increase the serum in the medium to 20% (vol/vol). For 10 ml of the cryopreservation medium, combine 8 ml of cell complete medium, 1 ml of DMSO and 1 ml of FBS. ▲ **CRITICAL** The medium should be freshly prepared before use.

EQUIPMENT SETUP

Biosafety cabinet (hood)—Turn on the UV light 30 min before performing the experiments.

Scissors and tweezers—Autoclave the scissors and tweezers before the experiment.

Stereomicroscope with swan-neck fiber optic illumination—Sterilize the microscope and the illumination source with 70% (vol/vol) ethanol solution before the experiment.

Phase-contrast microscope—Sterilize the microscope with 70% (vol/vol) ethanol solution before observing the cell clones.

Confocal laser microscope—The imaging system is an Olympus Fluoview FV1000 confocal laser microscope system. The system uses continuous-wave lasers at 405, 488 and 568 nm for fluorescence excitation.

Power supply for agarose gel electrophoresis—Set up the power supply to 100 V and to a running time of 30 min.

CytoVu/SiMPore thin membrane coculture system—Autoclave the slide before use and incubate it with cell culture medium overnight in the incubator before seeding the cells.

Sapphire disk preparation—Clean disks with detergent or 70% (vol/vol) ethanol and rinse them thoroughly with ddH₂O. Deposit a medium, dark film of carbon on the surface of the grid using a carbon coater. Scratch an asymmetric character (e.g., 'F') into the carbon layer to denote the sample side. Bake the disks overnight at 120 °C to secure the carbon film to the sapphire disks and prevent it from coming off in the culture dish. Secure disks (uncoated side down) to a culture dish with a small amount of agarose. Sterilize the entire assembly via UV or microwave before adding cells and medium.

PROCEDURE

Collection of the cochlear stria vascularis from young mouse ear ● TIMING ~1 h

- 1| Prepare anesthetic, fresh artificial perilymph solution, culture medium and collagen I-coated dishes before starting the experiment (Reagent Setup and Equipment Setup).
- 2| Weigh three P10–P15 C57BL/6J mice and anesthetize them with a dose of 0.1 ml per 20 g of body weight of anesthetic cocktail. Anesthetize the chest area topically with 1% lidocaine HCl at a concentration of 10 mg ml⁻¹, and euthanize the mice by intracardial injection with 0.2 ml of the euthanasia solution. Monitor the respiration and heart rate of the mice until both respiration and heart function cease.

 ! **CAUTION** The procedures involving mouse surgery and the use of mouse tissue must be reviewed and approved by your Institutional Animal Care and Use Committee. In the US, a DEA license is required.

 ▲ **CRITICAL STEP** Mice <P7 are too young, and at this age the stria vascularis has not yet separated from the spiral ligament. Choose P10–P15 animals for your experiments.
- 3| Swab the head and neck of the mouse with ethanol.

- 4| Decapitate mice with sharp scissors, and rapidly remove and place the auditory bullae in a Petri dish filled with cold, fresh artificial perilymph solution (the isolation medium is kept in the refrigerator at 4 °C).
 ▲ **CRITICAL STEP** Perform the surgery under sterile conditions.
- 5| Gently pull away the stria vascularis from the cochlear spiral ligament and place it in ice-cold perilymph solution. The isolation of the stria vascularis should be completed in less than 1 h.
 ▲ **CRITICAL STEP** The isolation solution should not be more than 1 week old. The isolation procedure should be performed as fast as possible to minimize cell damage.

7 TROUBLESHOOTING

Preparing mini-chip explants of cochlear stria vascularis from whole-mounted tissue ●

TIMING ~1 h

- 6| Wash the isolated stria vascularis by gentle shaking in cool perilymph solution for 10 min.
- 7| Transfer the stria vascularis into a clean 35-mm collagen I-coated dish with 2 ml of cell culture medium. (EC growth medium should be used for growing ECs. Similarly, PC and PVM/M growth medium should be used for growing PCs and PVM/Ms.)
- 8| Tear the stria vascularis into small 0.15–0.20-mm³ pieces with new and sterile ophthalmic tweezers under a dissection microscope.
 ▲ **CRITICAL STEP** Use new and sharp ophthalmic tweezers (size no. 5). Gently, evenly and in an orderly manner cut the stria vascularis from the apex to the basal end. In general, this procedure is completed in ~60 min.
- 9| Allow the fragmented pieces of stria vascularis to settle by gravity and reposition them to uniform density.
 ▲ **CRITICAL STEP** The tissue tends to stick to the tips of the forceps. Tissues should be grasped in the forceps about two-thirds of the way up from the tip.
 ▲ **CRITICAL STEP** Results will improve with the experimenter's experience. Improvement will be seen in the first 2 or 3 months of using this protocol.

7 TROUBLESHOOTING

Selective culture of phenotypes ● **TIMING 17–24 d**

- 10| Incubate the fragmented pieces of stria vascularis at 37 °C in 5% CO₂ with selective tissue growing medium.
 7 TROUBLESHOOTING
- 11| For production of different cell types, use different growth culture media (Reagent Setup).
 ▲ **CRITICAL STEP** For production of PCs, strictly control glucose concentration to 1 g per liter and PEDF at 100 nM. The tight control is necessary and essential for promoting PC growth and inhibiting EC growth. Strict quality

control is exercised in order to standardize the culture medium for each experiment. Contamination can cause variation.

- 12| Check on cell clone formation on the second day after seeding under a sterile phase-contrast microscope and change the first cell growth medium on the third day.
 ▲ **CRITICAL STEP** Do not change the medium in the first 2 d, and do not move the dish in the first 24 h.
- 13| When the cells reach 90% confluency, remove the medium and wash the cultures with PBS (three times for 5 min); follow this by incubation in trypsin-EDTA solution for 5 min at 37 °C and 5% CO₂. Pipette the cell suspension up and down a few times to detach the cell colony.
- 14| Passage the cells into a large and new (60 mm) collagen I-coated dish with a density of $1.5 \times 10^4 \text{ cm}^{-2}$ for ECs, $7.5 \times 10^3 \text{ cm}^{-2}$ for PCs and $1.5 \times 10^4 \text{ cm}^{-2}$ for PMV/Ms to promote uniform cell growth.
- 15| Check the cell growth every 2 d under a phase-contrast microscope and change the medium every 2 d for about 5–7 d (second passage). In general, cells grow to confluency in 5–7 d.
- 16| Passage the confluent cells into a second 60-mm collagen I-coated dish at a ratio of 1:2.
- 17| Change the culture medium every 2 d and grow the cells for ~5–7 d (third passage).

Validation of phenotype

- 18| Transfer the cells to a 35-mm collagen-coated glass-bottom dish at a density of $2.5 \times 10^4 \text{ cm}^{-2}$, and allow the cells to adhere for at least 24 h. Confirm each cell phenotype for the presence of two protein-specific markers. The markers are vWF and isolectin GS-IB4 from *Griffonia simplicifolia* (GS-IB4) for ECs, F4/80 and MiTF for PVM/Ms and desmin and PDGFR- β for PCs (option A). For validation of morphology, confirm each cell for the presence of F-actin and a specific protein marker, vWF for ECs, F4/80 for PVM/Ms and PDGFR- β for PCs (option B). Validate the detailed morphology of the three cell lines by SEM (option C). In addition to validation with immunofluorescence, analyze the cells for gene expression with RT-PCR (option D).

7 TROUBLESHOOTING

- A. Immunofluorescence ● **TIMING** 24 h (plus 2 h 30 min, plus overnight, plus 2 h on the following day)
 - i. Fix cells in 1 ml of 4% (vol/vol) PFA in 1× PBS (pH 7.2) for 15 min at room temperature.
 ! **CAUTION** PFA is a toxic reagent. Avoid inhalation, ingestion and skin contact. Perform cell fixation in a hood.
 - ii. Remove the 4% (vol/vol) PFA and wash the cells with 2 ml of PBS (three times for 10 min).
 - iii. Permeabilize the cells in 1 ml of 0.5% (vol/vol) Triton X-100 in PBS-BSA for 3 min at room temperature.

- iv. Remove the Triton X-100 solution and wash the cells with 2 ml of PBS (three times for 10 min).
 - v. Incubate the cells for 1 h in 1 ml of an immunofluorescence blocking solution at room temperature.
 - vi. Incubate the cells with primary antibodies diluted in a PBS-BSA solution overnight at 4 °C.
 - vii. Wash the cells with 2 ml of PBS (three times for 10 min).
 - viii. Incubate the cells with secondary antibodies diluted in PBS-BSA solution for 1 h at room temperature.
 - ix. Wash the cells with 2 ml of PBS (three times for 10 min).
 - x. Image the cells under a confocal laser microscope.
- B. Validation of morphology** ● **TIMING** 24 h (plus 2 h 30 min, plus overnight, plus 2 h on the following day)
- i. Fix the cells in 1 ml of 4% (vol/vol) PFA in 1× PBS (pH 7.2) for 15 min at room temperature.
! CAUTION PFA is a toxic reagent. Avoid inhalation, ingestion and skin contact. Perform cell fixation in a hood.
 - ii. Remove the 4% (vol/vol) PFA and wash the cells with 2 ml of PBS (three times for 10 min).
 - iii. Permeabilize the cells in 1 ml of 0.5% (vol/vol) Triton X-100 in PBS-BSA for 3 min at room temperature.
 - iv. Remove the Triton X-100 solution and wash the cells with 2 ml of PBS (three times for 10 min).
 - v. Incubate the cells for 1 h in 1 ml of an immunofluorescence blocking solution at room temperature.
 - vi. Incubate the cells with primary antibodies diluted in a PBS-BSA solution overnight at 4 °C.
 - vii. Wash the cells with 2 ml of PBS (three times for 10 min).
 - viii. Incubate the cells with secondary antibodies and Alexa Fluor 488–phalloidin diluted in PBS-BSA solution for 1 h at room temperature.
 - ix. Wash the cells with 2 ml of PBS (three times for 10 min).
 - x. Image the cells under a confocal laser microscope.
- C. SEM** ● **TIMING** ~32 h
- i. Clean the sapphire disk and tack the disk down with a drop of agarose in the well of a 35-mm collagen-coated glass-bottom dish. Sterilize the entire assembly via UV before adding the cells and medium.

- ii. Detach the cells as described in Step 14 with a solution of trypsin-EDTA. Add 200 μ l of cell suspension to the well at a concentration of $1 \times 10^5 \text{ ml}^{-1}$. Incubate the dish at 37 °C for a few hours until the cells stick to the disk, add the appropriate culture medium and incubate the dish again for at least 24 h.
- iii. Discard the culture medium and fix the cells (from the third passage) in 2.5% (wt/vol) glutaraldehyde/PBS solution.
! CAUTION This reagent is corrosive. Wear gloves and eye protection, and avoid inhalation of the reagent. Perform the fixation of the cells in a hood.
- iv. To remove the 2.5% (wt/vol) glutaraldehyde/PBS solution, wash the cells with PBS three times at room temperature (15 min per rinse), and then wash them with 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature (15 min per rinse).
! CAUTION Sodium cacodylate is a toxic reagent. Avoid inhalation, ingestion and skin contact. Perform cell fixation in a hood.
- v. Postfix cells in 1% (wt/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, at 4 °C.
! CAUTION Osmium tetroxide is a highly corrosive reagent. Wear gloves and eye protection, and avoid inhalation, ingestion and skin contact with the reagent. Perform the fixation of the cells in a hood.
- vi. To remove osmium tetroxide, wash the cells with 0.1 M sodium cacodylate (pH 7.2) at room temperature (15 min per rinse).
! CAUTION Sodium cacodylate is a toxic reagent. Avoid inhalation, ingestion and skin contact. Perform the fixation of the cells in a hood.
- vii. Remove water from the specimen with ethanol dehydration. Immerse the cells in gradually increasing concentrations of ethanol (35, 50, 70, 80, 95 and 100%, vol/vol) for 10 min each, repeating the final 100% incubation three times with fresh ethanol.
- viii. Trim a piece of double-sided carbon tape to fit your SEM stub.
- ix. Secure the bottom of your disk (sample side up) to the carbon-taped stub.
- x. Open the specimen chamber of the sputter coater (Polaron coating system) and load the samples at the center of the stub holder. Secure the chamber shut and confirm that the VENT and LEAK valves are closed.

- xi.** Start the pump by turning the four-position SELECTOR knob to PUMP.
- xii.** Open the main valve on the argon tank by turning a half-turn counterclockwise.
- xiii.** Check the vacuum meter and slowly open the VENT valve to flush the specimen chamber with argon gas once the pointer is between 10 and 20 mA. Once the VENT valve is open, wait for 3–5 s, and then close the valve completely.
- xiv.** Flush the chamber a second time by repeating Step 18C(xiii).
- xv.** Open the LEAK valve slowly until the vacuum meter reads 20 mA.
- xvi.** Begin the coating process and allow it to run for an appropriate length of time. Twelve seconds is a typically sufficient time, but this will vary based on the size of the sample.
- xvii.** Turn off the sputter coater pump and fully open the VENT valve.
- xviii.** Once you can open the chamber, close all the valves on the argon tank and remove your samples from the stub holder.
- xix.** Image the cells under a scanning electron microscope (Hitachi S-5000).

D. Gene identification with RT-PCR ● TIMING ~6 h

- i.** Detach the cells as described in Step 14 with a solution of trypsin-EDTA.
- ii.** Collect the cells in a 5-ml polypropylene round-bottom tube and centrifuge the cells at 300g for 5 min at 4 °C.
- iii.** Remove the supernatant and extract RNA from the pellet using the RNeasy micro kit according to the manufacturer's instructions.
- iv.** Synthesize cDNA using the RETROscript kit reverse transcription for RT-PCR, according to the manufacturer's instructions and by using the following reaction conditions: 44 °C for 1h; 92 °C for 10 min; then hold at 4 °C.
- v.** Prepare one master mix per gene of interest (*NG2*, *PDGFRβ* (*PDGFRB*), desmin (*DES*), *CD34*, *VWF*, *GSTα4* (*GSTA4*), and F4/80 (*EMRI*)) containing its forward and reverse primers and reagents in the RETROscript kit, according to the manufacturer's instructions.
- vi.** Run the sample PCR according to the following parameters:

Cycle number	Denature	Anneal	Extend	Hold
1	95 °C, 4 min			
2–41	95 °C, 30 s	60 °C, 30 s	72 °C, 45 s	
42			72 °C, 5 min	
43				4 °C

- vii. Run the products of the PCR in 1.5% (wt/vol) agarose gel (Reagent Setup and Equipment Setup).

Cell purity assessment with FACS ● TIMING ~3 h

- 19| Detach the cells as described in Step 14 using a trypsin-EDTA solution.
- 20| Gently wash the cells with 2 ml of culture medium and centrifuge at 300g for 5 min at 4 °C. Carefully aspirate the supernatant.
- 21| Resuspend the cell pellet to 1×10^7 cells ml⁻¹ in its own culture medium.
- 22| Incubate the cells in the dark with different antibodies for 30 min at 4 °C. For ECs, use lectin from *B. simplicifolia* (*G. simplicifolia*) (isolectin B₄ (BSI-B₄), FITC conjugate) at a concentration of 20 µg ml⁻¹. Lectin preincubated with 0.2 M galactose is used as control. For PCs, use phycoerythrin-conjugated rat mAb for PDGFR-β at a concentration of 0.5 µg ml⁻¹. Rat IgG phycoerythrin at a concentration of 0.2 µg ml⁻¹ serves as the isotype control. For PVM/Ms, use phycoerythrin-conjugated rat mAb for F4/80 at a concentration of 10 µl per 10⁶ cells (in 100 µl). Rat IgG phycoerythrin at a concentration of 0.2 µg ml⁻¹ serves as the isotype control.
 ▲ **CRITICAL STEP** Antibodies should be incubated in the dark at 4 °C.
- 23| Add 2 ml of culture medium to the cells and vortex and centrifuge the cells at 300g for 5 min at 4 °C. Discard the supernatant and wash the cells again in 1 ml of culture medium.
- 24| Resuspend the cell pellet in 500 µl of culture medium in a 5-ml polypropylene round-bottom tube for sorting.
 ▲ **CRITICAL STEP** Keep the cells on ice before sorting.
- 25| Analyze and sort the cells with a BD influx cell sorter at 45 p.s.i. using a 70-µm nozzle.
- 26| Centrifuge the positive cells at 300g for 5 min at 4 °C and discard the supernatant. Resuspend the cell pellet in prewarmed culture medium and place it in two 60-mm collagen I-coated dishes at a 1:2 split ratio. Return the dishes to the incubator. The cells will adhere to the bottom of the dishes and start to grow.

Cell storage (cryopreservation) ● TIMING 20–30 min plus overnight

- 27| Remove the medium from one dish; wash and trypsinize it as in Step 14.
- 28| Once the cells are detached, add 2 ml of medium to the dish and transfer the cells to a 5-ml polypropylene round-bottom tube.
- 29| Count the cells using trypan blue stain for a viable cell count.
 ▲ **CRITICAL STEP** The viability should be over 90% to ensure that the cells are healthy enough for freezing.

- 30| Spin down at 300*g* for 5 min at 4 °C and remove the medium.
 - 31| Resuspend the cells in enough cryopreservation medium to create a cell suspension of 1×10^6 cells per ml. Pipette the cells up and down to ensure a uniform mixture; aliquot 1 ml of the cell suspension into a cryogenic vial (in general, 1×10^6 cells per cryogenic vial is desired).
 - 32| Store the cryogenic vial in a Nalgene Cryo 1 °C freezing container overnight in a –86 °C freezer.
 - 33| Store the cryogenic vial in liquid nitrogen for long-term storage after Step 32.
- ▲ **CRITICAL STEP** Investigators should wear gloves, maintain hygienic conditions, use fresh plasticware when possible, autoclave the instruments and use sterile solutions.

7 **TROUBLESHOOTING**—Troubleshooting advice can be found in Table 1.

● TIMING

- Steps 1–5, collection of cochlear stria vascularis from young mouse ear: ~1 h
- Steps 6–9, preparation of mini-chip explants of stria vascularis from whole-mounted tissue: ~1 h
- Steps 10–17, selective culture of phenotypes: 17–24 d
- Step 18A, immunofluorescence: 24 h (plus 2 h 30 min, plus overnight, plus 2 h on the following day)
- Step 18B, validation of morphology: 24 h (plus 2 h 30 min, plus overnight, plus 2 h on the following day)
- Step 18C, SEM: ~32 h
- Step 18D, gene identification with RT-PCR: ~6 h
- Steps 19–26, cell purity assessment with FACS: ~3 h
- Steps 27–33, cell storage (cryopreservation): 1 d (20–30 min plus overnight)

ANTICIPATED RESULTS

We validated the phenotype of PCs, PVM/Ms and ECs by immunostaining for marker proteins and verifying gene expression with RT-PCR. Figure 3a–c shows DIC images showing cultured PCs, ECs and PVM/Ms at day 5 of the third passage, with Figure 3d–i showing the corresponding low- and high-magnification confocal images of each cell line immunostained with antibodies for marker proteins. Figures 3j–l show the RT-PCR gene analysis of each cell line.

Cell lines should be morphologically characterized and validated. The cell lines are phalloidin labeled for cytoskeleton protein (F-actin) and immunostained for marker protein. The cultured PCs, Figure 4a, are large, flat and stellate shaped with a broad filopod morphology, similar to that described by Shepro and Morel^{29–31}. The PVM/Ms (Fig. 4b) previously identified as melanocytes⁵, have unique dendritic processes³². ECs (Fig. 4c) have flat, elongated and generally cuboidal morphology, which is consistent with an early report³³. More details on the morphology of the three cell lines are shown in SEM (Fig. 4d–f).

Cell purity and cell line phenotype should be assessed and validated by FACS. The method provides high-quality ECs, PCs and PVM/Ms with a purity >90% after two passages (Fig. 5). Figure 5a–c shows DIC images of trypsin-treated detached cells at passage three before FACS analysis. Figure 5d–f shows confocal images of the detached cells with single marker labeling. The merged images of Figure 5g–i shows that the cultures have consistent single-color fluorescence emission and lack contamination from other cell lines. The purity of each cell line, validated by FACS, is shown in bivariate histograms (Fig. 5j–l). The mean purity from multiple FACS analyses is $94 \pm 2\%$ (ECs, mean $\pm$ s.e.m., $n = 6$), $93 \pm 3\%$ (PCs, mean $\pm$ s.e.m., $n = 5$) and $93 \pm 6\%$ (PVM/Ms, mean $\pm$ s.e.m., $n = 3$). Percentage loss in each cell line from FACS analysis is ~25% (Fig. 5m–o). Figure 5p–r shows images of purified PCs, ECs and PVM/Ms, plated and immunostained at day 5 with their marker proteins.

The primary cell lines enable direct study of intercellular interactions between ECs, PCs and PVM/Ms in coculture models. Figure 6 shows a schematic of three cell culture-based *in vitro* models, including cocultivated Transwells (Fig. 6a, Model I), conditioned medium coculture (Fig. 6b, Model II) and the CytoVu/SiMPore thin membrane coculture system (Fig. 6c, Model III). The interaction of ECs, PCs and PVM/Ms affects the expression of blood-labyrinth barrier tight-junction proteins. These cell culture-based *in vitro* models offer a unique opportunity to directly study the specific contribution of EC, PC and PVM/M signaling to blood-labyrinth barrier integrity, and to obtain a better understanding of the organ-specific characteristics of the cochlear blood-tissue barrier.

Acknowledgments

This work was supported by US National Institutes of Health (NIH) National Institute on Deafness and Other Communication Disorders (NIDCD) grant no. DC008888-02A1 (X.S.), NIH NIDCD grant no. DC008888-02S1 (X.S.), NIH NIDCD grant no. R01-DC010844 (X.S.), NIH NIDCD grant no. R21-DC12398-01 (X.S.), NIH grant no. P30-DC005983 and NIH National Institute of General Medical Services (NIGMS) grant no. P01-051487-15 (M.A.).

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Figure 1.

Outline of the steps in the explant procedure. (a) Six cochleae are needed to produce sufficient cells for each cell line. (b) An image of artificial cochleae emphasizes that six cochleae are required to produce a cell line at each trial. (c) The cochlear lateral wall is fully isolated from the cochlea and placed in fresh ice-cold perilymph solution. Scale bar, 500 μm . (d) Strips of cochlear stria vascularis are gently pulled away from the spiral ligaments in fresh ice-cold perilymph solution under a dissection microscope. (e) The stria vascularis and spiral ligament are shown to be fully separated. Scale bar, 500 μm . (f) Six strips of cochlear stria vascularis are 'banked' in fresh culture medium under an inverted microscope. Scale bar, 500 μm . (g) The fragmented pieces are plated to uniform density in 35-mm collagen I-coated dishes under an inverted microscope. SV, stria vascularis; SL, spiral ligament. Scale bars, 1,000 μm .

Figure 2.

Outline of the selective culturing procedure. (a) Growth media provide optimal conditions for selective growth of cochlear ECs, PCs or PVM/Ms. (a–c) Multiple cell clusters (red arrowheads) are seen to form around the explanted stria vascularis chips by day 1 or day 2, with cell clusters expanding (pink circles) and individual cells spreading (pink arrows) over the surface of the Petri dish by day 6. SV, stria vascularis. Scale bars, 100 μm.

Figure 3.

Validating the phenotype of PCs, PVM/Ms and ECs. (a–c) DIC images of cultured ECs, PCs and PVM/Ms at day 5 of the third passage. (d–f) PCs are triple-labeled for desmin (red), PDGFR β (green) and nuclei (blue), PVM/Ms for F4/80 (red), MiTF (green) and nuclei (blue), and ECs for von Willebrand factor (vWF, green), GS-IB4 (red) and nuclei (blue). (g–i) PCs, PVM/Ms and ECs are shown under high magnification with the same immunostaining. (j–l) show the RT-PCR gene analysis of each cell line.

Figure 4. Morphological validation of cell lines. (a–f) Confocal maximum projection images and scanning electron micrographs of PCs, PVM/Ms and ECs. (a) PCs triple-labeled for PDGFRβ (red), phalloidin (green) and nuclei (blue). (b) PVM/Ms triple-labeled for F4/80 (red), phalloidin (green) and nuclei (blue). (c) ECs triple-labeled for vWF (red), phalloidin (green) and nuclei (blue). (d–f) Scanning electron micrographs of each cell line, showing similar morphological features as the confocal images.

Figure 5.

FACS was used to assess cell purity and validate phenotype. (**a–c**) DIC images of the detached cell lines (third passage at day 5) before FACS. (**d–f**) Images of single color-stained cell lines: PCs with PE-PDGFR β , PVM/Ms with PE-F4/80 and ECs with FITC-BSI-B4. (**g–i**) Merged DIC and fluorescence images. (**j–l**) FACS results validate the purity. The purity of each cell line is >90%. (**m–o**) FACS validation incurs a cell loss on the order of 16% (PCs), 25% (PVM/Ms) and 19% (ECs). (**p–r**) Purified PCs are triple labeled for desmin (red), PDGFR β (green) and nuclei (blue); purified PVM/Ms are triple labeled for F4/80 (red), MiTF (green) and nuclei (blue); and purified ECs are triple labeled for von Willebrand factor (vWF, green), GS-IB4 (red) and nuclei (blue).

Figure 6.

The schematic illustrates several variations of the *in vitro* blood-labyrinth barrier model. **(a)** Model I shows a schematic of cells cocultivated on a Transwell layer. Images of an EC monolayer labeled with antibody for ZO-1 are shown as a monoculture and coculture setup. **(b)** The schematics and images in Model II show an EC monolayer labeled with antibody for ZO-1 and treated with different conditioning growth medium. **(c)** A schematic of the CytoVu/SiMPore thin membrane coculture system is shown in Model III. **(d)** The accompanying confocal fluorescence images show direct visualization of BSI-B₄-labeled live ECs on the thin membrane (left) and a population of PE-PDGFRβ-labeled PCs to one side of the thin membrane and FITC-BSI-B₄-labeled ECs on the other (right).

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Unable to obtain whole strips of stria vascularis	Bones in the cochlea of P10–P15 animals are soft and fragile. The procedure requires experience	Use the rough tips of tweezers (opposite the sharp tips) to follow the cochlear bony wall and gently remove the bone with small movements. Be extremely gentle
8, 9	Tissues adhere to the instrument	The small fragments are adhesive	Allow the edge of the fragment to stick to the tip of the forceps and quickly lift the tip out of the medium. The liquid surface tension will attract the tissue
10	Stria vascularis fragments do not adhere to the bottom of the dish	The dish was moved before the cells adhered	Do not move or change the medium in the first 2 d, especially in the first 24 h
	Cell clusters are not well formed	Reason not known	Use a collagen I-coated culture dish
18	PCs show a range of morphologies	The cells are at different stages: mature, immature and proliferating stages	Microscopic examination alone is not sufficient for validating cell phenotype. Cell types are accurately identified by specific marker proteins and RT-PCR gene analysis