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REGULAR ARTICLE

Structural changes in thestrial blood–labyrinth barrier of aged C57BL/6 mice

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Abstract Tight control over cochlear blood flow (CoBF) and the blood-labyrinth barrier (BLB) in the striavascularis is critical for maintaining the ionic, fluid and energy balance necessary for hearing function. Inefficient CoBF and disruption of BLB integrity have long been considered major etiologic factors in a variety of hearing disorders. In this study, we investigate structural changes in the BLB of the striavascularis in age-graded C57BL/6 mice (1 to 21 months) with a focus on changes in two blood barrier accessory cells, namely pericytes (PCs) and perivascular-resident macrophage-like melanocytes (PVM/Ms). Decreased capillary density was detectable at 6 months, with significant capillary degeneration seen in 9- to 21-month-old mice. Reduced capillary density was highly correlated with lower numbers of PCs and PVM/Ms. "Drop-out" of PCs and "activation" of PVM/Ms were seen at 6 months, with drastic changes being observed by 21 months. With newly established in vitro three-dimensional cell-based co-culture models, we demonstrate that PCs and PVM/Ms are essential for maintaining cochlear vascular architecture and stability.

Keywords Strial blood–labyrinth barrier · Pericyte perivascular resident macrophage-like melanocytes · Confocal laser microscopy · Mouse (C57BL/6J)

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Introduction

Age-related hearing loss (also known as presbycusis) is a major form of hearing loss, characterized by progressive deterioration in auditory sensitivity, loss of auditory sensory cells and ultimately in changes in central processing functions. Age-related deterioration in the striavascularisis is one of the prominent causes of age-related hearing loss (Schulte and Schmiedt 1992; Gratton et al. 1996, 1997; Spicer and Schulte 2002; Ohlemiller et al. 2008; Ohlemiller 2009).

The high metabolic demands of sound transduction requires blood-labyrinth barrier (BLB) integrity to sustain the ionic and metabolic homeostasis essential for hearing (Juhn and Rybak 1981; Suzuki et al. 1998; Juhn et al. 2001; Cohen-Salmon et al. 2007; Laurell et al. 2008). Disruption of the BLB affects cochlear homeostasis, disrupts endocochlear potential (EP) and leads to hair cell damage (Harkins 1981; Brown et al. 1995; Gratton et al. 1996, 1997; Seidman et al. 1999; Schacht and Hawkins 2005; Ohlemiller et al. 2008, 2010; Frisina 2009; Lang et al. 2010).

BLB in the striavascularis, in the classic view, is composed of endothelial cells (ECs) and an underlying basement membrane (BM; Sakagami et al. 1982, 1987). Endothelial cells connect to each other by tight junctions (Sakagami et al. 1982; Takeuchi et al. 2001) and form a diffusion barrier that selectively excludes most blood-borne substances from entering the ear, thereby protecting it from systemic influences (Juhn

and Rybak 1981; Juhn 1988). In a recent study, the BLB has been discovered to include, in addition to ECs and BM, a large number of pericytes (PCs; Takeuchi et al. 2001; Shi et al. 2008; Shi 2009) and perivascular resident macrophage-like melanocytes (PVM/Ms; Shi 2010; Zhang et al. 2012, 2013a, 2013b; Neng et al. 2013a, 2013b). PCs and PVM/Ms are new classes of cells in the BLB and their function is largely uncharacterized.

PCs are multi-potent mesenchymal-like cells primarily located on microvessels, vital for vascular development, blood flow regulation, vascular integrity, angiogenesis and tissue fibrogenesis (Diaz-Flores et al. 1991; Balabanov and Dore-Duffy 1998; Allt and Lawrenson 2001; Betsholtz et al. 2005; Dore-Duffy et al. 2006; Peppiatt et al. 2006; von Tell et al. 2006; Quaegebeur et al. 2010; Greenhalgh et al. 2013; Hall et al. 2014). PC pathology, leading to vascular dysfunction, is seen in many diseases, including stroke, heart infarct and retinal diseases (Puro 2007; Liu et al. 2012; Greenhalgh et al. 2014; Greif and Eichmann 2014; O'Farrell and Attwell 2014). In particular, loss of PCs attributable to retinopathy and Alzheimer's disease has been highly associated with capillary degeneration (Mendel et al. 2013; Sagare et al. 2013). Cochlear PCs, rich in the structural protein desmin (Shi et al. 2008), wrap around the capillaries and are thought to give mechanical strength and generally to enhance the integrity of capillary networks in the striavascularis (Shi et al. 2008). Blood vessels deficient in PCs are abnormally large and leaky (Hellstrom et al. 2001). Abnormal cochlear PCs have been shown to cause vascular leakage in loud-sound-damaged vasculature (Shi 2009).

The stria capillary network is also surrounded by PVM/Ms, a hybrid cell type with characteristics of both macrophage and melanocyte (Zhang et al. 2012). Similar to the structures of the blood brain barrier (BBB) and blood-retinal barrier (BRB), the strial BLB comprises ECs, PCs, PVM/Ms and BM (Shi 2011; Zhang et al. 2012). Complex intercellular tight junctions between ECs limit the passive diffusion of molecules into the cochlea and result in blood vessels exhibiting extremely low permeability, excluding most blood-borne substances from entering the ear (Juhn and Rybak 1981; Juhn 1988). PCs are embedded in BM in which they communicate with ECs through both direct physical contact and paracrine signaling (Shi et al. 2008; Neng et al. 2013a). PVM/M-end-feet, which are structurally and functionally similar to the end-feet of brain astrocytes (Neng et al. 2013a; Zhang et al. 2013a), provide physical support and facilitate signal communication between ECs and PCs (Zhang et al. 2012). PVM/M signaling regulates the expression of tight junction (TJs) and adherens junction protein, with loss or deformation of PVM/Ms associated with barrier breakdown, tissue edema and drop in the EP (Zhang et al. 2012, 2013a).

Capillary destruction and regression in aged animals has long been observed. For example, Prazma and co-workers (1990) reported decreased cochlear blood flow (CoBF) in old gerbils compared with young animals. Gratton et al. (1996) reported vascular abnormalities (atrophic capillaries) in the striavascularis of aged gerbils. Changes in whole blood viscosity and red-cell rigidity have also been correlated with high-frequency hearing loss in elderly human subjects (Gatehouse and Lowe 1990). Increased immunoglobulin and laminin deposits have been observed in the thickened BMs of aged strial capillaries (Sakaguchi et al. 1997a, 1997b) and dramatic degeneration of the strial capillaries has been found in aged diabetic congenic mice (Ohlemiller et al. 2008). Whereas much is known about age-related vascular pathology in general, particular pathologies of PCs and PVM/Ms in aged animals has not yet been directly studied. In this report, we demonstrate the age-associated population and morphological changes in PCs and PVM/Ms. With a newly established in vitro three-dimensional (3-D) matrix gel model, we show that PCs and PVM/Ms are essential for angiogenesis and vascular stability, suggesting that PC and PVM/M pathology might be essential factors contributing to BLB deterioration in aged animals.

Materials and methods

Animals

Animals were purchased from Jackson Laboratory (Bar Harbor, Me., USA): male C57BL/6 mice (stock number: 000664) and NG2DsRedBAC transgenic mice (stock number: 008241000664). The C57BL/6 males and NG2DsRedBAC transgenic mice were divided into three age groups: young adult (1 and 3 months), middle-aged (6, 9, and 12 months) and old adult (15, 18, and 21 months). All procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon Health & Science University (approval number: MU7_IS00001157).

Transmission electron microscopy

Cochleae from mice of various ages were isolated for transmission electron microscopy (TEM). Segments of the cochlear lateral wall from the basal turns were fixed overnight in phosphate-buffered 3 % glutaraldehyde-1.5 % paraformaldehyde and post-fixed in 1 % osmium. Tissues were dehydrated through a graded alcohol series, embedded in Embed 812 (Electron Microscopy Sciences, Hatfield, Pa., USA), sectioned, stained with lead citrate (Electron Microscopy Sciences) and uranyl acetate (Electron Microscopy Sciences) and viewed on a Philips EM 100 transmission electron microscope (Philips/FEI Corporation, Eindhoven, Holland).

Immunohistochemistry and fluorescence microscopy

Eight groups of mice of various ages were killed. The cochleae were harvested and fixed in 4 % formaldehyde overnight at 4 °C and then rinsed in 37 °C phosphate-buffered saline (PBS; pH 7.3) to remove any residual formaldehyde. Immunohistochemistry was performed as previously described (Shi 2009). Tissue samples were permeabilized in 0.5 % Triton X-100 (Sigma, St. Louis, Mo., USA) for 1 h and then immunoblocked for 1 h with a solution of 10 % goat serum (Sigma) and 1 % bovine serum albumin (Fisher Scientific, Pittsburgh, Pa., USA) in 0.02 mol/l PBS. The specimens were incubated overnight at 4 °C with primary antibodies (Table 1), namely with a rabbit monoclonal to detect platelet-derived growth factor recepetor-β (PDGFR-β; Abcam, Cambridge, Mass., USA) and with a rat monoclonal antibody to detect F4/80 (eBioscience, San Diego, Calif., USA). Primary antibodies were diluted 1:50 in PBS-bovine serum albumin. After three washes in PBS, the samples were incubated with secondary antibodies, namely Alexa Fluor 568 goat anti-rabbit IgG (H + L) from Life technologies (Eugene, Ore., USA) or Fluor 488 goat anti-rat IgG (H + L) from Life technologies. Controls were prepared by replacing primary antibodies with PBS. Either isolectin *Griffonia simplicifolia*-IB4 (GS-IB4) Alexa Fluor 568 conjugate or Alexa Fluro 488 conjugate (Life technologies) diluted in PBS-bovine serum albumin at 1:100 was used to label the capillaries for an additional 1 h at room temperature. The tissues were mounted in mounting medium (H-1000; Vector Laboratories, Burlingame, Calif., USA).

To assess PC and PVM/M number and capillary density, PCs in the striavascularis of mice at various ages (cohorts of three) were counted on a standard epifluorescence microscope (Olympus, Tokyo, Japan) with a 40× objective. For each cochlea, counts were obtained at three randomly chosen, non-overlapping locations for each turn (apex, mid, and base) along the 300-μm length of the striavascularis. The vessels and striavascularis were traced manually with a free line and area tool in Olympus Fluoview Ver. 1.7b software and the length of the capillaries and area of the striavascularis were measured by means of the same program. PC, PVM/M and capillary density were calculated as follows: PC density $= \frac{\text{number of PCs}}{\text{length of capillary(mm)}}$; PVM/M density $= \frac{\text{number of PVM/Ms}}{\text{area of stria vascularis(mm2)}}$; and capillary density $= \frac{\text{length of capillary(mm)}}{\text{area of stria vascularis (mm2)}}$. Images were captured on an FV1000 Olympus laser-scanning confocal microscope (Olympus).

EC, PC and PVM/M co-culture and measurement of endothelial tubule formation and angiogenesis

Mouse ECs, PCs and PVM/Ms were isolated and purified as described previously (see Neng et al. 2013b). The purified PCs were transfected with pmOrange2-N1 Vector (Clontech Laboratories, Mountain View, Calif., USA) and the fluorescent signal was visualized at 568 nm laser excitation. PVM/Ms were transfected with Pegfp-N2 Vector (Clontech Laboratories) and the fluorescent signal was visualized at 488 nm excitation. ECs were transfected with pE2-Crimson-N1 Vector (Clontech Laboratories) and the fluorescent signal visualized at 647 nm excitation.

The effect of PCs and PVM/Ms on angiogenesis and vascular stability was assessed in vitro with 3-D matrigel models. Four different culture models, namely EC only, EC + PC, EC + PVM/M and EC + PC + PVM/M, were designed for the study. All three cell types were fluorescently labeled by genetic encoding: ECs with PE2-Crimson, PCs with pmOrange2 and PVM/Ms with pEGFP-N2. The 3-D matrigel, prepared from 130 μl ice-cold matrigel matrix (BD Biosciences, Franklin Lakes, N.J., USA), was added to each well of a LabTek chambered cover-glass (Thermo Scientific Nunc, Waltham, Mass., USA) by using chilled pipettes and allowed to polymerize for at least 30 min at 37 °C in a 5 % CO_2 incubator. For the EC group, a 100-μl EC suspension (3.0×10^5 cells/ml) was applied to the 3-D matrix gel. For the EC + PC group, ECs were pre-seeded on the matrix gel for 6 h and then a 50-μl PC suspension at a concentration of 3.0×10^5 cells/ml was added to the matrix for an additional 56 h. For the EC + PVM/M group, ECs were pre-seeded on the matrix gel for 6 h and then a 50 μl PVM/M suspension at a

Table 1 Applied antibodies (*PDGFR* platelet-derived growth factor recepetor-β, *GS-IB4 Griffonia simplicifolia*-IB4, *BSA* bovine serum albumin, *PBS* phosphate-buffered saline, *PVM/M* perivascular-resident macrophage-like melanocytes, *PC* pericytes)

Primary antibodies	Supplier	Catalog number	Dilution with 1 % BSA in PBS	Type	Application
Anti-F4/80	eBioscience	14-4801-85	1:50	Rat monoclonal antibody to F4/80	Labeling PVM/M
Anti-PDGFR-β	Abcam	Ab32570	1:100	Rabbit monoclonal to desmin	Labeling PC
GS-IB4 Alexa Fluor 568 conjugate	Life technologies	I21412	1:100	Fluorescent dye	Labeling capillary

concentration of 1.5×10^5 cells/ml was added to the matrix for an additional 56 h. For the EC + PC + PVM/M group, a 100-μl EC suspension at a concentration of 3.0×10^5 cells/ml was pre-seeded on the matrix gel for 6 h and then a 50 μl PVM/M + PC suspension at concentrations of either 1.5×10^5 cells/ml or 3.0×10^5 cells/ml was added to the matrix for an additional 56 h. EC tube formation was visualized on an FV1000 Olympus laser-scanning confocal microscope and images were captured at various time points. The number of capillary-like EC tubes was quantified by counting branching points per area. Angiogenesis was calculated by using Image J as the quantified area (%) = $\frac{\text{area of sprouting}}{\text{area of EC-tube}}$.

Statistics

Data are presented as means±SD. Statistical analyses included linear correlation and one-way analysis of variance, followed by a Tukey multiple comparison test (GraphPad Prism 5.0). A 95 % confidence level was considered statistically significant.

Results

Reduction in capillary density seen with ageing correlates with loss in PCs and PVM/Ms

The number of PCs and PVM/Ms on capillaries of the striavascularis at the apical, middle and basal turns was assessed by fluorescence immunohistochemistry combined with confocal microscopy. PCs were labeled with an antibody for the marker protein, PDGFR-β. PVM/Ms were labeled with an antibody for F4/80. Capillaries were stained with either isolectin GS-IB4 Alexa Fluor 568 or isolectin GS-IB4 Alexa Fluor 488. In this study, we also visualized PCs by detecting a bright red fluorescence variant (NG2DsRed.T1) under the control of a neural/glial antigen 2 (NG2) Cspg4 promoter (Hall et al. 2014). NG2 is specifically expressed in strial PCs (Shi et al. 2008). PC distribution in the strial capillaries at various ages was easily and clearly visualized by both immunofluorescence labeling and genetic-encoded fluorescence signals.

Our results showed no significant differences in PC or PVM/M density between apical, middle and basal turns in young mice. Aged animals, however, exhibited a significant decrease in PC and PVM/M density in all regions (see Fig. 1). For example, 1-month-old animals had a PC density of 21±2 / mm capillary, whereas 21-month-old animals had a density of 13±1 / mm capillary (Fig. 1a). The density of PVM/Ms also significantly dropped. The 1-month-old animals showed a PVM/M density of 352±39 / mm^2 strial area, whereas at 21 months, a density of 247±35 / mm^2 strial area was recorded in all turns (Fig. 1b). We also found that strial capillary density was significantly reduced in 6-month-old mice compared with 3-month-old mice and was markedly lower in 9-month-old and 21-month-old mice.

Morphology of PCs and PVM/Ms in aged animals

NG2 is specifically expressed in cochlear PCs (Shi et al. 2008). PC distribution and morphology adjacent to strial capillaries were easily visualizedin transgenic NG2DsRedBAC mice at various ages by using confocal microscopy (Fig. 2a–c). In young animals (at <3 months), PCs exhibiting a flat and slender morphology were in normal abundance and tightly associated with ECs. In older animals (>6 months), however, PCs were less abundant and exhibited an altered morphology. PCs in the older animals had a prominent round body morphology in less physical contact with the endothelium, as previously described as being a sign of PC migration (Pfister et al. 2008). Electron microscopy confirmed the abnormal character of the PCs. Those in young animals had a prominent nucleus and small cytoplasm and were tightly associated with the abluminal surface of the endothelium (Fig. 2d). In aged animals, PCs showed a loss of cytoplasmic organelles and had a vacuolated appearance detached from ECs (Fig. 2e).

Morphology of PVM/Ms in aged animals

PVM/M morphology dramatically changed in aged animals. Figure 3a–c shows PVM/M morphology and distribution at the apical, middle and basal turns in the various age groups. In younger animals, most PVM/Ms exhibited a branched morphology. The cells were arranged in a “self-avoidance” pattern (Fig. 3a). However, in animals at 6, 9, 12 and 21 months, the PVM/Ms became smaller with shorter processes (Fig. 3b, c). In some regions, the PVM/Ms become flat and amoeboid-shaped (Fig. 3c) and exhibited less physical contact with the capillaries.

The biochemistry of the PVM/Ms also significantly changed. For example, PVM/Ms in younger animals did not display a terminal galactopyranosyl group on their membrane surface. PVM/Ms in aged animals (>6 months), however, had an exposed terminal galactopyranosyl group on their membrane surface, as detected by binding to the lectin GS-IB4, a hallmark of macrophage activation.

Under TEM, PVM/Ms in young animals showed a trace of melanin in the cytoplasm (Fig. 3d). By contrast, PVM/Ms in aged animals contain a significantly greater amount of melanin (Fig. 3e). Taken together, the data showed that PVM/Ms were discernibly affected by ageing.

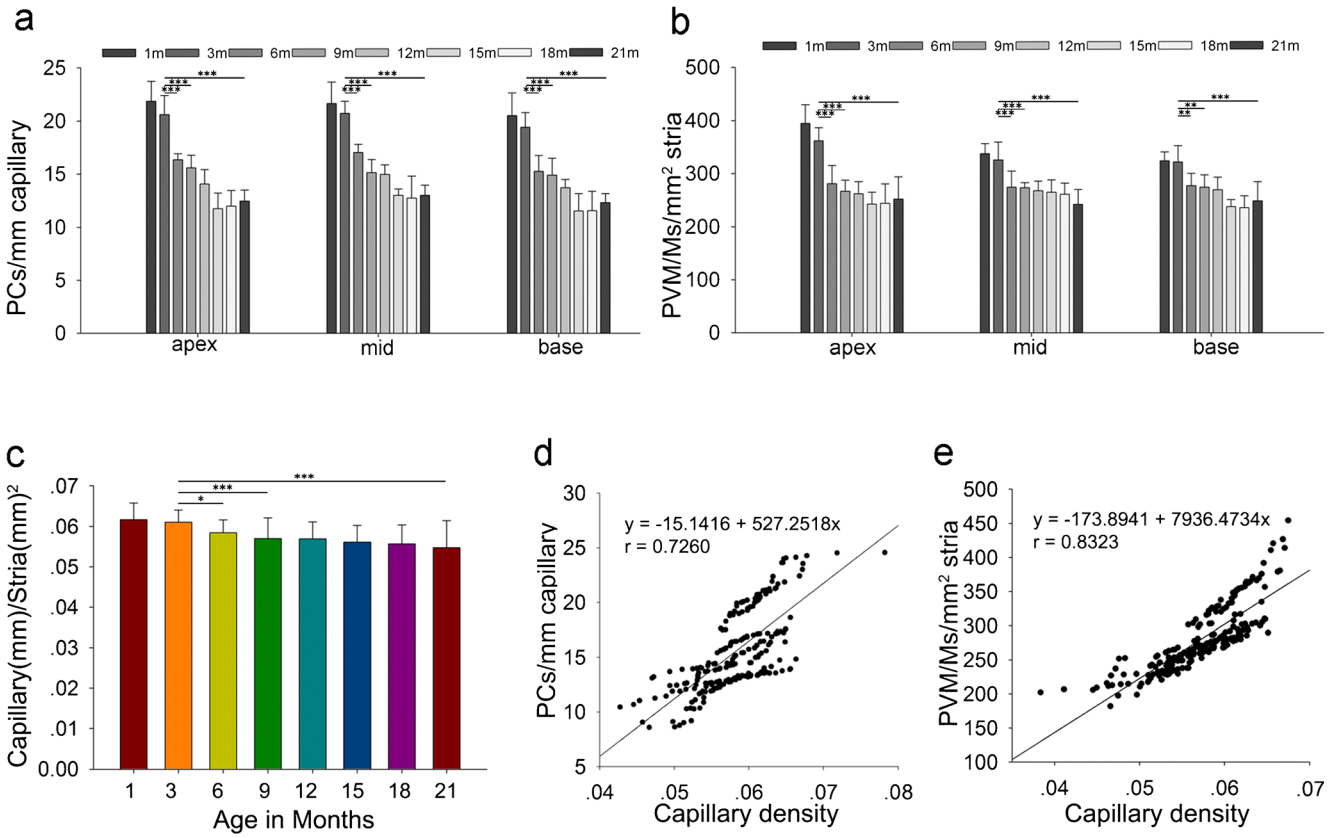

Fig. 1 **a** Mean density of pericyte (*PC*) population at the apical, middle and basal turns in C57BL/6 mice at various ages. PCs are uniformly distributed on the strial capillary with no distinction between regions. However, PC density progressively decreases with age in all regions. Significant differences are found at 6, 9 and 21 months compared with at 3 months [$F_{apical(7,64)}$=68.7,***P<0.0001,***$P_{apical\ (6m/9m/21m\ vs\ 3m)}$< 0.001; $F_{middle\ (7,64)}$=62.78, ***P<0.0001, ***$P_{middle(6m/9m/21m\ vs\ 3m)}$< 0.001; $F_{basal\ (7,64)}$=45.6, ***P<0.0001, ***$P_{basal\ (6m/9m/21m\ vs\ 3m)}$< 0.001]. **b** Mean value of the perivascular-resident macrophage-like melanocytes (PVM/M) population at the apical, middle and basal turns in animals at various ages. A significant and progressive reduction in the PVM/M population across regions is found at 6, 9 and 21 months compared with at 3 months [$F_{apical\ (7,64)}$=31.73, ***P<0.0001, ***$P_{apical\ (6m/9m/21m\ vs\ 3m)}$<0.001; $F_{middle\ (7,64)}$=17.20, ***P<0.0001, ***$P_{middle\ (6m/9m/21m\ vs\ 3m)}$<0.001; $F_{basal\ (7,64)}$=17.35, ***P<0.0001, **$P_{basal\ (6m/9m\ vs\ 3m)}$<0.01,***$P_{basal\ (21m\ vs\ 3m)}$<0.001]. **c** Density of strial capillaries is statistically lower in 6-month-old mice than that in 3-month-old mice and shows a marked decrease at 9 and 21 months [$F_{stria\ (7,424)}$=16.67, ***P<0.0001, *$P_{stria\ (6m\ vs\ 3m)}$<0.05, ***$P_{stria(9m/21m\ vs\ 3m)}$<0.001]. **d**, **e** Loss of PCs [$r_{(216)}$=0.73, ***P<0.0001] and PVM/Ms[$r_{(216)}$=0.83, ***P<0.0001] and reduction in capillary density are linearly correlated

Morphology of BM in aged animals as shown by TEM

TEM analysis revealed an abnormal BM in aged animals. A dense and continuous BM was seen between PCs and ECs in young animals under high magnification (Fig. 4a). In contrast, the BM was loose and less electron-dense in aged animals (Fig. 4b).

PCs and PVM/Ms are essential for vessel stability and angiogenesis

We tested whether PCs and PVM/Ms were required for stability and sprouting of new blood vessels (angiogenesis) in artificially created capillary networks in a 3-D matrigel model. The two histograms in Fig. 5 demonstrate a significant difference in the stability of capillaries and degree of angiogenesis in the presence and absence of PCs and PVM/Ms (Fig. 5a, b). PC-induced angiogenesis was first observed at 32 h in culture, with remarkable sprouting angiogenesis seen by 56 h (Fig. 5a). PVM/M stabilization of EC-tube formation was noticeable at 26 h in culture, with dramatic changes observed by 56 h (Fig. 5b).

Figure 5c-j presents representative images showing that EC forms sparsed capillary-like networks around 11 h (Fig. 5c–f) and 56 h (Fig. 5g–j). ECs forming capillary-like networks had significantly regressed by 26 h but had mostly regressed by 56 h when ECs were alone in culture (Fig. 5g). Co-culture of ECs with PCs induced a marked degree of sprouting angiogenesis, as shown in Fig. 5h. Similarly, a slow retrogression of capillary networks was seen in the EC and PVM/M co-culture (Fig. 5i). ECs, PCs and PVM/Ms in triple culture promoted both capillary stability and angiogenesis (Fig. 5j). Our in vitro model demonstrates PCs and PVM/Ms are both essential for sustaining normal capillary architecture.

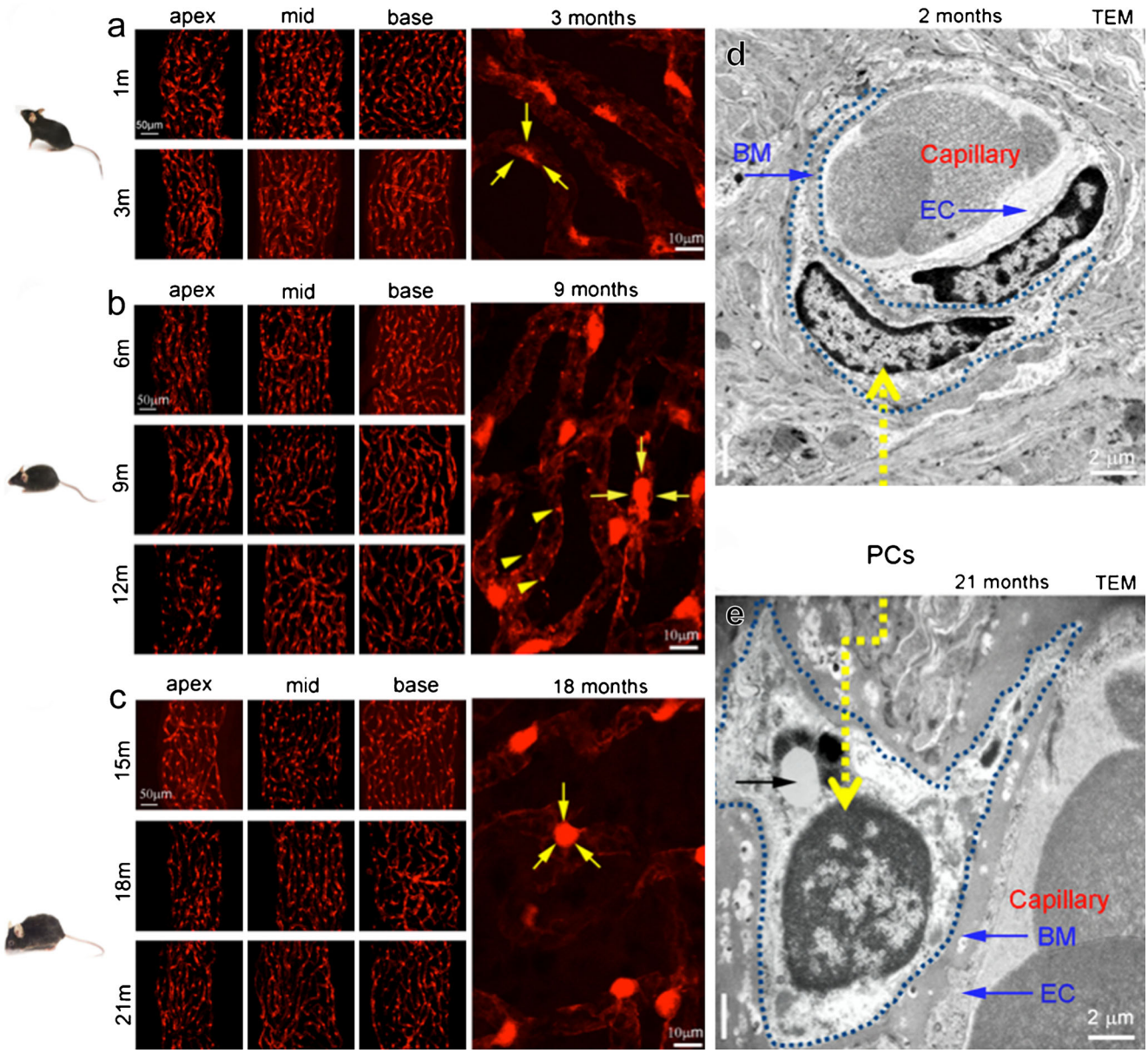

Fig. 2 PC morphology in young and aged animals; fluorescent confocal microscopy and transmission electron microscopy (TEM). **a** Confocal image exhibiting the flat and slender cell body of PCs distributed in various regions (*left* apical, *middle* middle, *right* basal) in a young animal (transgenic mice with fluorescently labeled NG2, *red*). **b**, **c** PC morphology changes in middle- and old-aged animals. The PC shows a "prominent round" cell body (*arrows*) in less physical contact with the strial capillary at the apical (*left*), middle (*middle*) and basal (*right*) turns. **d** Transmission electron micrograph of cochlear PCs at <3 months. PCs appear as long and slender polymorphic cells located on the abluminal side of the endothelial cell (*EC*; *yellow arrow*). **e** Irregularly shaped PCs of a 21-month-old mouse; note the sparse caveolae (*BM* basemsnt membrane)

Discussion

Microvascular structure and function are key aspects of tissue and organ health. Ageing is accompanied by a progressive failure in normal cell and organ function. In this study, we characterize the pathological changes in strial capillary networks in three age-graded sets of C57BL/6 mice, with particular focus on the changes in PCs and PVM/Ms. We demonstrate cochlear-organ-specific pathological changes in capillary PCs and PVM/Ms in animals with presbycusis. The PCs tend to "drop-out", whereas the PVM/Ms tend to "activate" with age. A decreased population of PCs and PVM/Ms is highly correlated with reduced capillary density. With newly established in vitro cell-based models, we demonstrate PCs and PVM/Ms are essential for vascular architecture and stability in the ear.

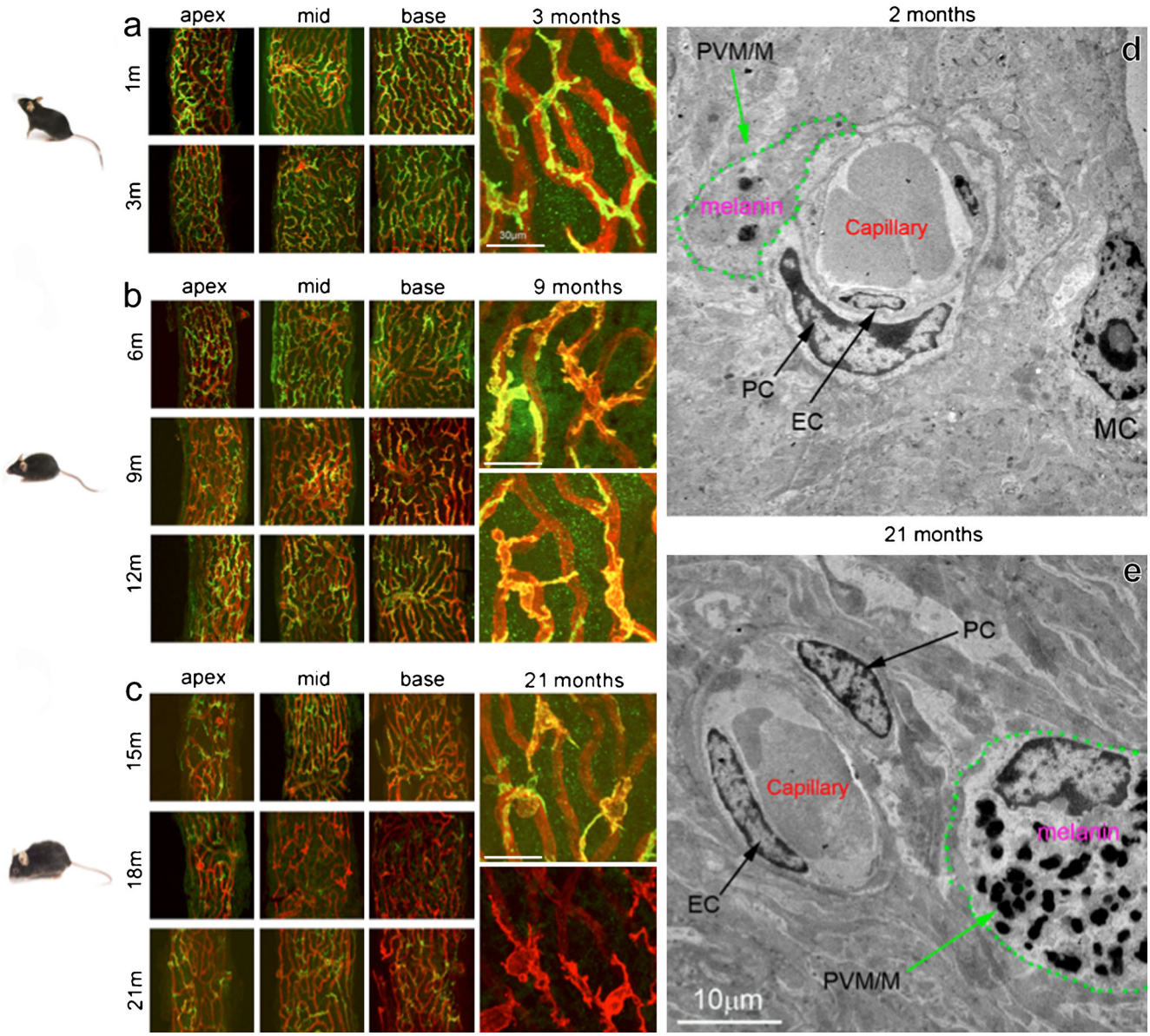

Fig. 3 PVM/M morphology in young and aged animals under fluorescent confocal microscopy and TEM. **a** Confocal image showing perivascular-resident macrophage-like melanocyte (*PVM/M*) morphology and distribution on strial capillaries in a young animal; PVM/Ms are labeled with antibody for F4/80 (*green*) and strial capillaries with *Griffonia simplicifolia*-IB4 (GS-IB4; *red*). PVM/M exhibit a long branched morphology and are distributed at the various cochlear turns (*left* apical, *middle* middle, *right* basal). **b** PVM/Ms in middle-aged animals show reduced branching and withdrawal of ramifications at the different turns (*left* apical, *middle* middle, *right* basal). **c** PVM/Ms in the old animals have much shorter processes and less physical contact with strial capillaries at the different turns (*left* apical, *middle* middle, *right* basal). The PVM/Ms are also positive for GS-IB4, an indication of activation. **d** Transmission electron micrographs of cochlear PVM/Ms at 2 months showing a flat cell body and cells containing a modest amount of melanin (*MC* melanocyte). **e** PVM/Ms at 21 months appear dark, because of the abundance of melanin in the cytoplasm

Structural changes in intra-strial fluid-blood barrier with changes in PCs and PVM/Ms

Age-related hearing loss, the most common form of hearing loss, is the predominant neurodegenerative disease of aging. The etiology of age-related hearing loss is not simple, as it is associated with a combination of genetic and environmental factors that result in multiple structural and functional abnormalities including changes in cochlear blood flow, effects on auditory nerves and brain processing of speech and sound and damage to sensory hair cells (Schacht and Hawkins 2005; Frisina 2009; Ohlemiller 2009; Ohlemiller et al. 2010).

Normal function of the cochlear microcirculation is critically important for nutrient supply and sustaining the EP in the inner ear is essential for the signal transduction of sound

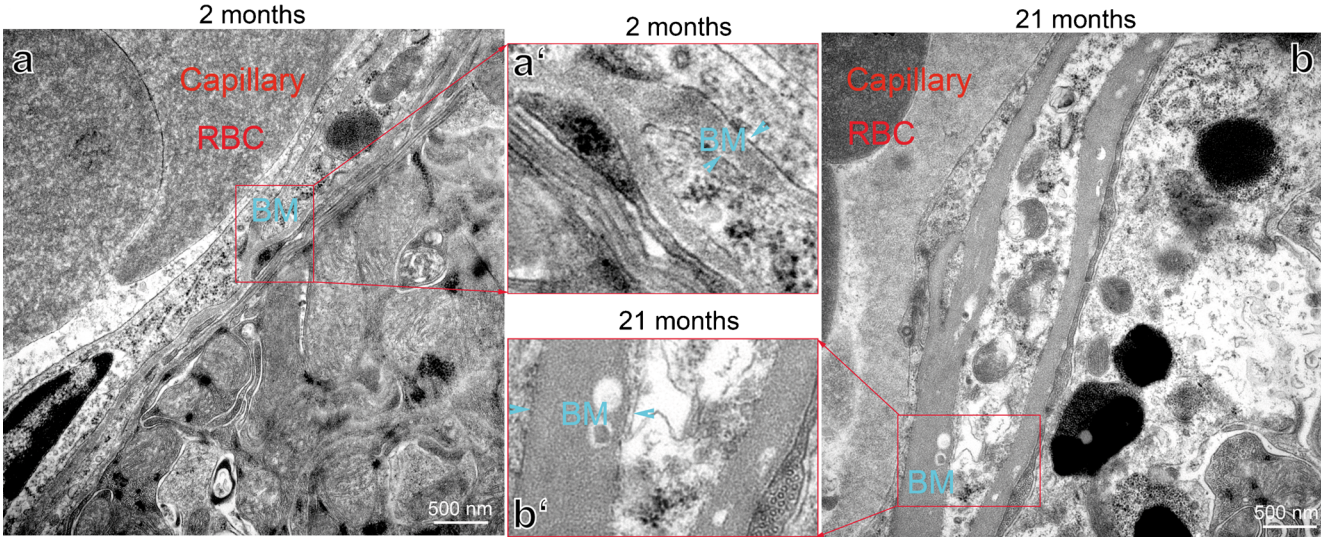

Fig. 4 Transmission electron micrographs of cochlear vessels from young and aged mice. **a** A fine and homogenous basement membrane (*BM*) with relatively high electron density is seen between normal PCs and ECs in the young mouse (2 months old). The *red inset* (**a'**) is a higher magnification view of uniform BM. **b** Thick and diffuse electron-dense BM with high porosity is observed in a 21-month-old mouse (area within *red rectangle* in **b** is shown at higher magnification in **b'**)

(Offner et al. 1987; Shi 2011). Dysfunction (insufficiency) of CoBF and disruption of the integrity of the BLB in the striavascularis (vascular leakage) engender an ischemic or hypoxic microenvironment, causing a shortage of nutrients and a harmful accumulation of metabolites in cochlear cells. Dysfunction of blood flow also causes structural instability in the tissue and cell loss.

Capillary density starts to decrease at age 6 months in the C57BL/6 mouse. Significant capillary degeneration is seen at 9 and 21 months. Accompanying the degeneration, the number of blood barrier accessory cells, namely PCs and PVM/Ms, are significantly reduced in aged animals. PCs are reduced by 38 % in 21-month-old mice and PVM/Ms by 26 %. Loss in PCs and PVM/Ms is highly correlated with reduced capillary density. In addition to the drop in cell numbers, morphologically abnormal PCs and PVM/Ms are also seen at 6 months. In particular, a large percentage of PVM/Ms in the aged animals are activated and morphologically changed. The PVM/Ms display reduced branching and withdrawal of ramifications and strong binding to GS-IB4, the latter being a sign of cell activation (Maddox et al. 1982).

PCs have a critical role in vascular stability and integrity, angiogenesis and regulation of capillary blood flow (Lindahl et al. 1998; Hellstrom et al. 1999; Peppiatt et al. 2006; Armulik et al. 2010; Daneman et al. 2010; Wang et al. 2014). PC deficiency is known to cause blood barrier breakdown and hypo-perfusion, contributing to tissue pathology and organ disease (Armulik et al. 2010; Daneman et al. 2010; Crawford et al. 2013; Wang et al. 2014). PCs also exhibit multi-potent stem cell activity and are found to differentiate into a variety of cell types, including macrophages, phagocytes, fibroblasts and smooth muscle cells (Sims 2000; Dore-Duffy et al. 2006). Various vascular diseases such as Alzheimer's disease, diabetic retinopathy and neurodegenerative disorders are associated with PC pathology and deficiency (Hellstrom et al. 2001; von Tell et al. 2006; Beltramo and Porta 2013; Sagare et al. 2013). Strial capillaries in the cochlear lateral wall are richly populated by PCs (Shi et al. 2008). The PCs are distributed in a regional- and tissue-specific manner, with the density reflecting the specific function of the micro-vessel and being tightly coupled to metabolic demand (Hirschi and D'Amore 1996; Sims 2000; Aguilera and Brekken 2014). In particular, PC-EC crosstalk promotes the formation of EC tight junctions (Daneman et al. 2010). In addition, PCs express high levels of intermediate filament proteins, including desmin. The abundant PC coverage of strial capillaries suggests the PCs have an important role in contributing to strial vessel stability and in controlling BLB integrity and function. Abnormally low numbers of PCs in the aged ear could lead to vascular instability and malfunction. Why PCs are lost in the aged animal is unknown and the exact mechanism underlying PC loss has not yet been determined. Our data, however, suggest that PCs present a novel therapeutic target for the mitigation of age-related hearing loss.

PVM/Ms, the resident macrophages of the striavascularis, have a critical role in maintaining tissue homeostasis and BLB integrity (Zhang et al. 2012). We previously found that PVM/Ms are activated in animals exposed to loud sounds or induced to experience inflammation (Zhang et al. 2013a, 2013b). Similar astrocyte activation is seen in brain ischemic diseases (Caruso et al. 2013). Is PVM/M "activation" also elicited by hypoxia? PVM/Ms are a hybrid of macrophage and

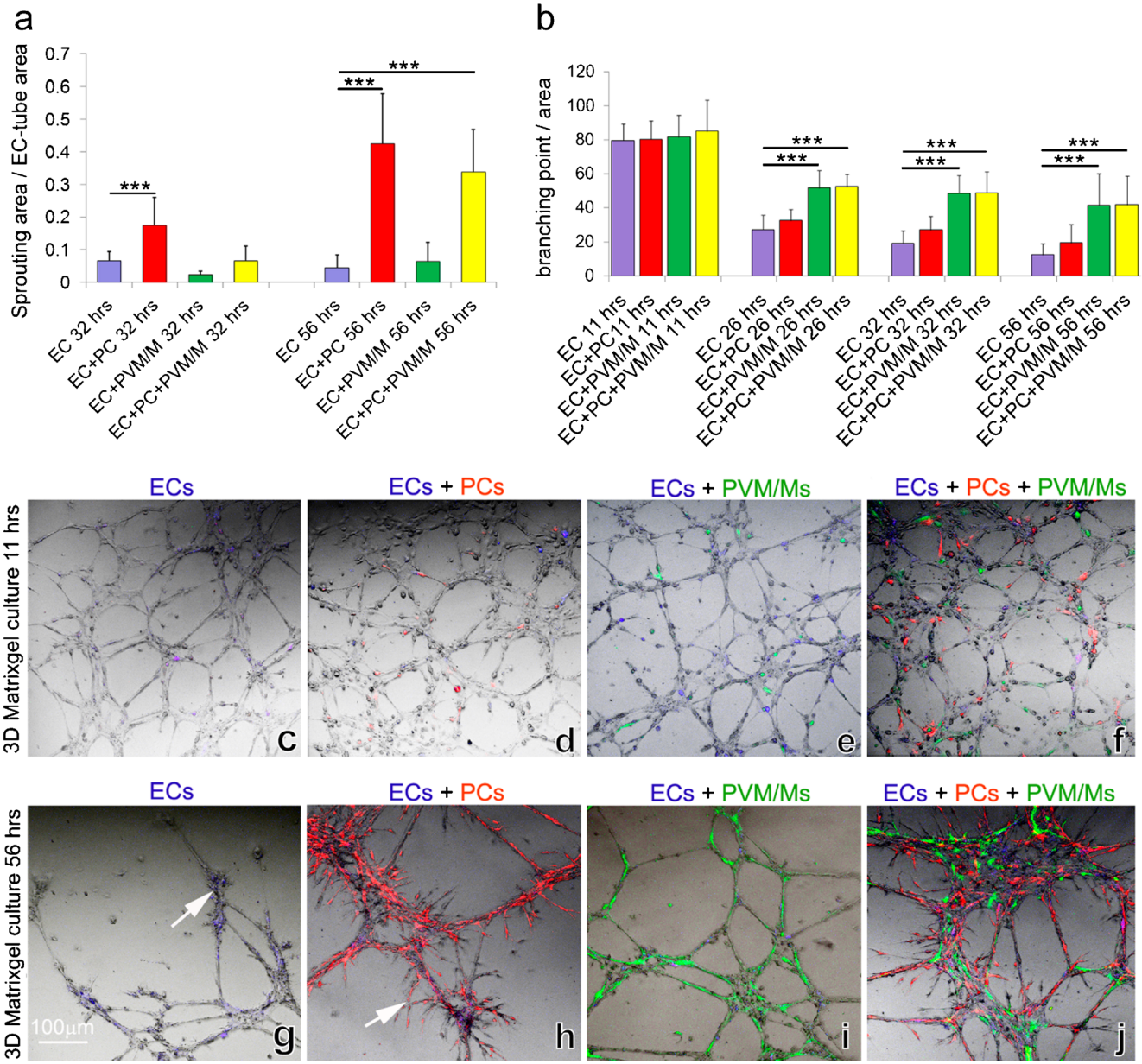

Fig. 5 Mono-culture of ECs and co-culture of ECs + PCs, ECs + PVM/Ms and ECs + PCs + PVM/Ms at various times in a three-dimensional (3-D) matrix gel. **a** PCs significantly promote sprouting angiogenesis [$F_{32h(3,31)}$=14.61, ***P<0.001; ***$P_{EC\ vs\ (EC+PC)\ 32h}$ <0.001, $P_{EC\ vs\ (EC+PVM/M)\ 32h}$ >0.05, $P_{EC\ vs\ (EC+PC+PVM/M)\ 32h}$ >0.05]; [$F_{56h\ (3,31)}$= 28.21, ***P<0.001; ***$P_{EC\ vs\ (EC+PC)\ 56h}$ <0.001, $P_{EC\ vs\ (EC+PVM/M)\ 56h}$ >0.05, ***$P_{EC\ vs\ (EC+PC+PVM/M)\ 56h}$ <0.001]. **b** PVM/Ms stabilize EC-formed capillary-like tubes [$F_{11h\ (3,25)}$=0.046, P>0.05; $P_{EC\ vs\ (EC+PC)\ 11h}$ >0.05, $P_{EC\ vs\ (EC+PVM/M)\ 11h}$ >0.05, $P_{EC\ vs\ (EC+PC+PVM/M)\ 11h}$ >0.05]; [$F_{26h\ (3,31)}$=21.41, ***P<0.001; $P_{EC\ vs\ (EC+PC)\ 26h}$ >0.05, ***$P_{EC\ vs\ (EC+PVM/M)\ 26h}$ <0.001, ***$P_{EC\ vs\ (EC+PC+PVM/M)\ 26h}$ <0.001]; [$F_{32h\ (3,31)}$= 20.15, ***P<0.001; $P_{EC\ vs\ (EC+PC)\ 32h}$ >0.05, ***$P_{EC\ vs\ (EC+PVM/M)\ 32h}$ <0.001, ***$P_{EC\ vs\ (EC+PC+PVM/M)\ 32h}$ <0.001]; [$F_{56h\ (3,31)}$=9.70, ***P<0.001; $P_{EC\ vs\ (EC+PC)\ 56h}$ >0.05, ***$P_{EC\ vs\ (EC+PVM/M)\ 56h}$ <0.001, ***$P_{EC\ vs\ (EC+PC+PVM/M)\ 56h}$ <0.001]. **c–j** Representative confocal images of a mono-culture of ECs and co-culture of ECs + PCs, ECs + PVM/Ms and ECs + PCs + PVM/Ms at 11 h and 56 h in a matrix gel. No effect on angiogenesis and EC tube regression is seen at 11 h in culture (**c–f**). Significant effects on angiogenesis and EC tube formation are seen at 56 h (**g–j**). **g** Sparse branched networks and sprouting angiogenesis are seen in the EC alone group at 56 h (*arrow*). **h** PCs promote sprouting angiogenesis of tube structures (*arrow*). **i** PVM/Ms significantly delay regression of EC-formed capillary-like tubes. **j** Both PCs and PVM/Ms are required for capillary stability and angiogenesis. The PCs and PVM/Ms promote sprouting angiogenes

melanocyte and melanocytes are responsive to biological and physicochemical signals in the local environment (Sulaimon and Kitchell 2003; Slominski 2009). In the aged animals, we found increased melanin secretion by the PVM/Ms. Melanin is known to buffer calcium, scavenge heavy metals and promote antioxidant activity (Drager 1985; Murillo-Cuesta et al. 2010; Slominski et al. 2012). The increased melanin pigment is likely to occur in response to noxious

factors produced in the aged animals. The cause of PVM/M pathology and the precise signal pathway for tissue-resident macrophage activation remain to be investigated.

Aged animals also show abnormal BM. Electron dense and continuous BM is displayed in the strial capillaries of young animals, whereas aged animals show less electron density in the BM and a looser association between PCs and ECs. The results corroborate earlier studies reporting abnormalities in the BM of aged animals (Gratton et al. 1996).

Deficiency of PCs and PVM/Ms causes structural changes and degeneration of BLB

Capillary networks are not static but are dynamically remodeled over time in response to various stimuli. The stability of the vascular networks entails a continual state of angiogenesis, which is the growth of new blood vessels from pre-existing vessels; this is a robust mechanism and includes vascular network remodeling that occurs in the embryonic stage that remains active throughout adulthood (Scianna et al. 2013). The developing animal requires angiogenesis during organ growth and development. The adult animal requires angiogenesis for re-establishing oxygen supply in wound healing and aged-related degeneration (Scianna et al. 2013).

A significant correlation was found between the population of PCs and PVM/Ms and capillary density in the aged animals, suggesting that the presence of PCs and PVM/Ms is essential for maintaining the stability of strial capillary networks. In order to determine whether PCs and PVM/Ms are required for vascular stability, we established an in vitro 3-D matrix gel cell-based model to assess the contribution of PCs and PVM/Ms to strial capillary degeneration. We particularly focused on the interaction between ECs, PCs and PVM/Ms in angiogenesis and vascular stability. The in vitro model enabled the identification of specific cell types that regulate these events. Our results show PC recruitment to have a significant effect on angiogenesis, with sprouting angiogenesis occurring in the presence of PCs. PVM/Ms in the model system were found to delay EC-tube retrogression and to promote vessel stability. The presence of both PCs and PVM/Ms promote angiogenesis and the stability of capillary-like networks. Our findings with the in vitro model support accumulating evidence that PCs play an important function in angiogenesis (Cappellari and Cossu 2013) and confirm the role of PVM/Ms in stabilizing micro-vessels (Zhang et al. 2012).

Conclusions and future work

Our data provide early evidence that abnormalities in PCs and PVM/Ms disrupt cochlear vascular integrity and lead to a reduction in the microvasculature. The exact mechanisms underlying these processes in C57BL/6 mice remain unknown, with the key mechanisms involved in cochlear microcirculation pathology still needing to be determined. Vascular function in many diseases is recognized as a clinically important therapeutic target. Targeting cell-mediated dysfunction of the peripheral microcirculation in hearing disorders, in particular in aging-related hearing loss, is important for the clinical restoration of hearing function. Further progress in this area should lead to a better understanding of cell-mediated BLB homeostasis and should lay the foundation for clinical studies that address the dysfunction of peripheral microcirculation-related hearing disorders.

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