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Identification of Vascular Endothelial Side Population Cells in the Choroidal Vessels and Their Potential Role in Age-Related Macular Degeneration

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Purpose. The neovascular form of age-related macular degeneration (AMD) is characterized by the growth of abnormal new blood vessels from the choroid, termed choroidal neovascularization (CNV). The origin of the new vessels in CNV, however, has not been elucidated fully to our knowledge. The purpose of this study is to identify vascular endothelial side population (SP) cells in the preexisting choroidal vessels, and investigate their potential role in AMD.

Methods. We made single cell suspensions of freshly isolated mouse choroidal, retinal, and brain tissue by enzymatic digestion. Vascular endothelial SP cells were isolated using flow cytometry based on the ability to efflux the DNA-binding dye, Hoechst 33342, via ATP-binding cassette (ABC) transporters.

Results. In the choroid, 2.8% of $CD31^{+}CD45^{-}$ vascular endothelial cells (ECs) showed a typical SP staining pattern. They were not bone marrow–derived and possessed high colony-forming capacity in vitro. They proliferated during laser-induced CNV in vivo. In contrast, stereotypic SP staining pattern was not observed in retinal and brain ECs. Retinal and brain EC-SP cells included increased SP populations with less colony-forming capacity within the SP compartment, because they contained cells with and without proliferative potential. The latter still could efflux the dye due to high levels of ABC transporters, such as ABCB1a, ABCC4, and ABCC6.

Conclusions. The EC-SP cells in the choroid may represent vessel-residing endothelial stem/progenitor cells contributing mainly to angiogenesis, and may be useful for augmenting vascular regeneration or for developing new antiangiogenic therapy in AMD.

Keywords: angiogenesis, endothelial cell, stem cell, side population, choroid

Ocular neovascular diseases, including diabetic retinopathy and the neovascular form of age-related macular degeneration (AMD), are the most common cause of severe vision loss worldwide.[1–3] Diabetic retinopathy is characterized by retinal ischemia accompanied by abnormal neovessel growth from the retinal vessels (retinal neovascularization),[1] whereas neovascular AMD is attributed to abnormal neovessel growth from the choroidal vessels (choroidal neovascularization [CNV]).[2,3] Retinal neovascularization and CNV are caused either by angiogenesis, a process involving new vascular endothelial cell (EC) sprouting from preexisting blood vessels,[4] or by vasculogenesis, involving bone marrow (BM)-derived circulating endothelial progenitor cells (EPCs) contributing to the neovasculature.[5–8] However, recent studies have suggested that the contribution of EPCs to neovessel formation is not as marked as reported previously.[9–12] Therefore, the main cellular origin of new ECs seems to be the vascular ECs residing in the preexisting blood vessels during angiogenesis-based neovessel formation.[11]

Vascular ECs residing in the preexisting blood vessels have been regarded as cells possessing equal potential to produce ECs in response to the angiogenic stimuli VEGF and bFGF. However, we recently identified a small population of vascular endothelial stem/progenitor cells in the preexisting blood vessels, which may be a source of new ECs during angiogenesis.[13] These cells were isolated using flow cytometry based on the side population (SP) phenotype, a common feature of adult stem cells, including hematopoietic, epidermal, and muscle stem cells characterized by the ability to efflux the DNA-binding fluorescent dye, Hoechst 33342, via ATP-binding cassette (ABC) transporters.[14–16] In contrast to the majority of cells that accumulate Hoechst 33342, termed main population (MP) cells, SP cells appear as a discrete unstained population to the side of the MP cells in a flow cytometry density dot plot. An SP cell population within the vascular ECs (EC-SPs) has been isolated from blood vessels in limb muscle.[13] Compared to non–EC-SP cells, EC-SP cells possess high proliferative potential in vivo and in vitro, consistent with their stem cell properties. However, EC-SPs in the retinal and choroidal vessels, and their role in angiogenesis have not been identified to our knowledge. In addition, EC-SP cells in the brain also have not been investigated in detail. In our study, we compared EC-SP cells in

the choroidal, retinal, and brain vessels to identify the origin of angiogenesis in those vessels.

Materials and Methods

Mice

The C57BL/6 mice and C57BL/6-Tg (CAG-EGFP) mice (EGFP mice) that express green fluorescent protein (GFP) in all tissues were purchased from Japan SLC (Shizuoka, Japan). Mice 8 to 12 weeks of age were used for experiments. All animal experiments were conducted in accordance with the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Preparation

Mice were euthanized and eyes were extracted. Retinal tissue was removed gently from the RPE–choroid–sclera complex. Choroidal tissue subsequently was scraped off the sclera. The whole brain also was extracted from the same mice. Respective tissue was excised, minced, and digested with Dispase II (Godo Shusei Corp., Chiba, Japan), collagenase (Wako, Osaka, Japan), and type II collagenase (Worthington Biochemical Corp., Lakewood, NJ) at 37°C.[17] The digested tissue was passed through 40-μm filters to yield single cell suspensions. Erythrocytes were lysed with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2-EDTA).

Flow Cytometry

Hoechst staining was performed as described previously.[14] Briefly, cell surface antigen staining was performed, and cell suspensions were incubated with Hoechst 33342 (5 μg/mL; Sigma, St. Louis, MO) at 37°C for 90 minutes in Dulbecco's modified Eagle's medium (DMEM, 2% fetal calf serum, 1 mM HEPES; Sigma) at a concentration of 10^6 nucleated cells/mL in the presence or absence of verapamil (50 μmol/L; Sigma). Cell surface antigen staining was performed as described previously.[18] The monoclonal antibodies (mAbs) used in immunofluorescence staining were anti-CD45 and anti-CD31 mAbs (eBiosciences, San Diego, CA). Respective isotype controls (eBiosciences) were used as negative controls. Propidium iodide (PI, 2 μg/mL; Sigma) was added before fluorescence-activated cell sorting (FACS) analysis to exclude dead cells. The stained cells were analyzed and sorted by a SORP FACSAria (BD Biosciences, San Diego, CA), and data were analyzed using FlowJo Software (Treestar Software, San Carlos, CA).

EC Colony-Forming Assay

The 10^3 EC-SP or MP cells were seeded on 24-well plates and cocultured on OP9 stromal cells in RPMI (Sigma), supplemented with 10% fetal calf serum (FCS) and 10^{-5}M 2-ME (Gibco, Grand Island, NY).[19] Cells were cultured for 10 days and the number of colonies counted after immunostaining.

Immunofluorescence

The procedure for staining was as reported previously.[20] For immunofluorescence, anti-CD31 mAb (BD Biosciences) was used for staining, and anti-rat IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA) and biotin-conjugated polyclonal anti-rat Ig (Dako, Glostrup, Denmark) were used as the secondary antibodies. Biotinylated secondary antibodies were developed using ABC kits (Vector Laboratories, Burlingame, CA). Cell nuclei were visualized with Hoechst dye (Sigma). Samples were visualized using an Olympus IX-70 equipped with UPlanFI ×4/0.13 and LCPlanFI ×20/0.04 dry objectives (Olympus Corporation, Tokyo, Japan). Images were acquired and processed with Adobe Photoshop CS3 software (Adobe Systems, Inc., San Jose, CA). All images shown are representative of more than four independent experiments.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from $CD31^+CD45^-$ EC cells, $CD31^+CD45^-$ EC-SP cells, and $CD31^+CD45^-$ EC-MP cells from the brain, retina, and choroid, respectively, using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was generated using reverse transcriptase from the ExScript RT reagent Kit (Takara, Otsu, Japan) as described previously.[21] Real-time PCR was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA). The polymerase chain reaction was performed on cDNA using specific primers (Supplementary Table S1). Expression level of the target gene was normalized to the GAPDH level in each sample.

Laser-Induced CNV

The C57BL/6 mice were anesthetized as described previously.[22] A total of 20 photocoagulation lesions was made with a diode laser (150 mW, 0.05 seconds, 75 μm; Ultima 2000 SE; Lumenis, Santa Clara, CA) between the retinal vessels in a peripapillary distribution in each fundus. Production of a subretinal bubble at the time of laser treatment confirmed the disruption of Bruch's membrane. The $CD31^+CD45^-$ ECs from the choroid were obtained 6 days after the laser procedure. Proportions and numbers of EC-SP cells per choroid were analyzed and calculated. Controls were the choroid from the untreated eye, or choroid from normal wild-type mice.

Murine BM Transplantation Model

The 8- to 12-week-old C57BL/6 mice underwent BM transplantation from same-aged EGFP donors. Briefly, BM cells were obtained by flushing the tibias and femurs of age-matched donor EGFP mice. The transplantation was performed into C57BL/6 mice lethally irradiated with 10.0 Gy, by intravenous infusion of approximately 1×10^7 donor whole BM cells. At 24 weeks after transplantation, by which time BM of recipient mice was reconstituted, the mice were used for the experiments. The percent reconstitution of the BM was confirmed in all mice at the time of experiments.

Statistical Analysis

All data are presented as mean ± SEM. For statistical analysis, SigmaStat software (SPSS, Inc., Chicago, IL) was used. When two groups were compared, a 2-sided Student's *t*-test was used. A probability value of less than 0.05 was considered statistically significant.

Results

Identification of Endothelial SP Cells in the Choroid, Retina, and Brain

We performed Hoechst 33342 staining and flow cytometric analysis of cells isolated from normal mouse choroid, retina, and brain to identify EC-SP cells. In the choroid, among cells positive for the EC marker CD31 and negative for the hematopoietic cell (HC) marker CD45 ($CD31^+CD45^-$ ECs, Fig. 1A), 2.8 ± 0.14% showed a typical SP staining pattern (i.e., Hoechst 33342 dye efflux properties, lost in the presence of the drug efflux pump inhibitor, verapamil, Fig. 1B). They were

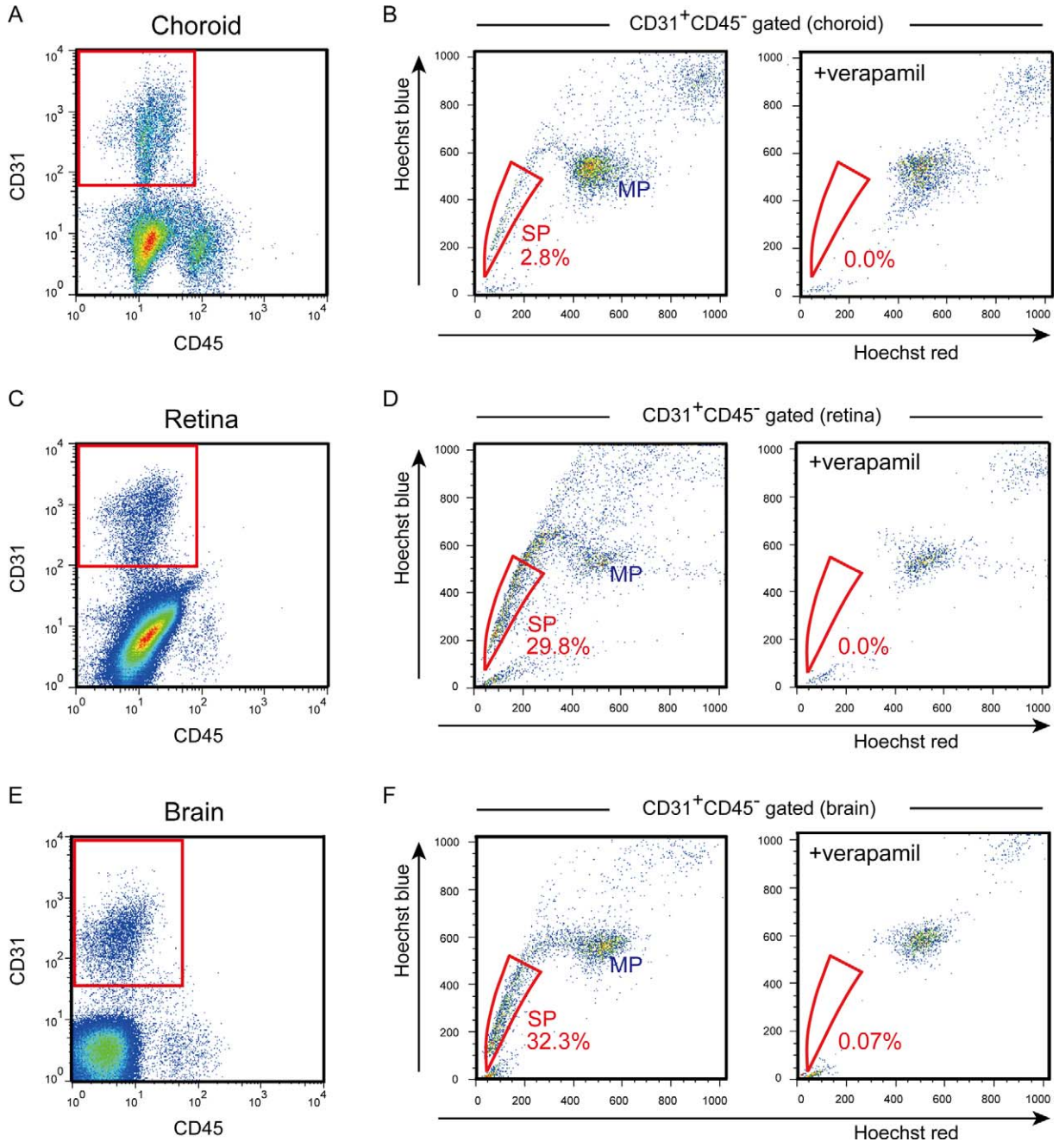

Figure 1. Identification of endothelial side population cells in the choroid, retina, and brain. (**A**) Flow cytometric analysis of choroidal ECs from wild-type mice. (**B**) Hoechst 33342 staining of $CD31^{+}CD45^{-}$ ECs gated as shown in (**A**). Of the cells, 2.8 ± 0.14% were in the SP gate. Note that verapamil selectively prevents Hoechst exclusion from EC-SP cells. (**C**) Flow cytometric analysis of retinal ECs from wild-type mice. (**D**) Of the cells, 29.8 ± 2.2% were in the SP gate. Note that a typical EC-SP pattern is not observed. (**E**) Flow cytometric analysis of brain ECs from wild-type mice. (**F**) Of the cells, 32.5 ± 2.6% were in the SP gate. Note the similar SP pattern as in the retina. Verapamil selectively prevents Hoechst exclusion from EC-SP cells.

distinct from the MP cells. On the other hand, the stereotypic SP staining pattern was not observed, and a higher proportion of SP cells were identified among $CD31^{+}CD45^{-}$ ECs from retina and brain compared to the choroid. Indeed, 29.8 ± 2.2% were in the SP gate in the retinal ECs (Fig. 1C) and 32.5 ± 2.6% in the brain (Fig. 1E). These cells also possessed Hoechst 33342 dye efflux properties, which disappeared in the presence of verapamil (Figs. 1D, 1F).

Proliferation and Colony-Forming Capacity of EC-SP Cells In Vitro

To evaluate the proliferative capacity of EC-SP cells in vitro, sorted EC-SP cells isolated from the choroid, retina, and brain were cultured on OP9 stromal cells, which support EC growth.[15] After 10 days, EC-SP cells isolated from the choroid generated a higher number of EC colonies compared to EC-MP cells (Fig. 2A). Each colony had a cord-like

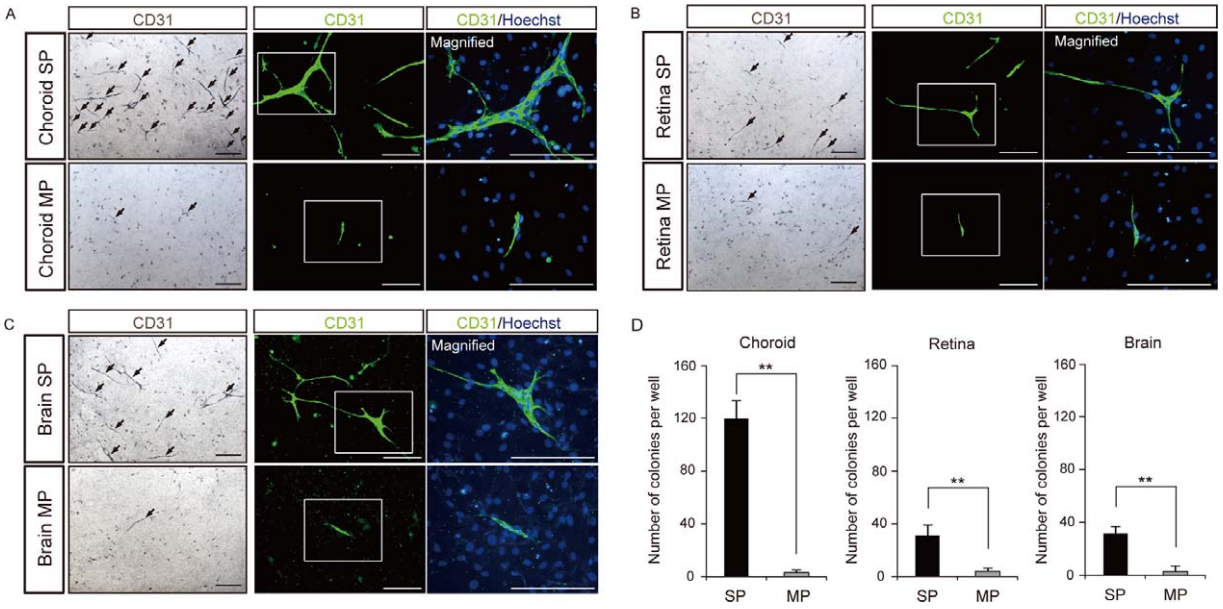

FIGURE 2. Endothelial SP cells in the choroid have high EC colony-forming ability. (**A**) EC-SP cells (*upper*) and EC-MP cells (*lower*) in the choroid were cultured on OP9 feeder cells and stained with anti-CD31 mAb. *Arrows* on the *left* image indicate each colony. Images on the *right* show a higher magnification of the areas indicated by boxes in the *middle* image. Note that EC-SP cells generate many CD31-positive EC colonies compared to EC-MP cells. Endothelial colony formation by EC-SP cells, or EC-MP cells from retina (**B**) or brain (**C**). Note that EC-SP cells (*upper*) in the retina (**B**) and brain (**C**) form many colonies, compared to EC-MP cells (*lower*), but the difference is less prominent compared to the choroid. (**D**) Quantitative evaluation of the number of CD31-positive ECs in one well of a 24-well culture dish. *Error bars* are ±SEM. $^{**}P < 0.01$ ($n = 4$). *Scale bars*: 500 μm.

structure, and included multiple ECs, as confirmed by CD31 and Hoechst staining. The EC-SP cells isolated from the retina and brain also formed higher numbers of ECs with a cord-like structure compared to respective EC-MP cells (Figs. 2B, 2C), but had substantially lower colony-forming ability than the EC-SP cells from the choroid (Fig. 2D).

EC-SP Cells Are Not Derived From Bone Marrow, and Are Distinct From EPCs

To confirm that EC-SP cells are not identical to EPCs, we transplanted BM cells from GFP mice into irradiated wild-type mice. Although the average percent reconstitution of the BM was more than 99% at 24 weeks after transplantation, as confirmed by flow cytometry (Figs. 3A–C), we could not detect any GFP-positive EC-SP cells among the $CD31^{+}CD45^{-}$ ECs from the choroid, retina, or brain of GFP BM-transplanted mice, suggesting that EC-SP cells do not originate from EPCs derived from BM (Figs. 3A–C). This indicates that EC-SP cells reside at the preexisting vessels in each tissue.

Expression of ABC Transporters in Choroidal, Retinal, and Brain ECs

Because the retinal and brain ECs had high proportions of EC-SP cells, but less efficient colony-forming capacity compared to choroidal ECs, we hypothesized that not only the stem cell–like ECs, but also nonstem cell–like ECs in the retina and brain also express high levels of ABC transporters constitutively to maintain the blood–retinal barrier (BRB) or blood–brain barrier (BBB). Therefore, we compared the ABC transporter gene family mRNA expression in choroidal, retinal, and brain ECs. Retinal and brain ECs showed similar expression patterns of several ABC transporters except for ABCC3 (Fig. 4). The expression levels of ABCB1a (multiple drug resistance 1a [MDR1a]), ABCA5, ABCC4, and ABCC6 were significantly higher in retinal and brain ECs compared to choroidal ECs (Figs. 4B, 4E, 4I, 4J). On the other hand, ABCB1b and ABCA9 were lower in retinal and brain ECs (Figs. 4C, 4G), indicating that they are not associated with an SP phenotype in these ECs. The relative expression of ABCG2, which is reported to correlate with the SP phenotype, was found to be high in the brain ECs (Fig. 4A). ABCG2 also tended to be highly expressed in the retinal ECs compared to choroidal ECs, but the difference was not statistically significant (Fig. 4A). These data suggested that components of the ABC transporters that maintain the BBB and BRB generally are similar, but different from the ECs in the choroid, which are distinguished from those in the brain and retina by the expression pattern of ABC transporter genes.

Next, we sorted the EC-SP and EC-MP cells from the brain, retina, and choroid, and compared the expression levels of several ABC transporters (Fig. 5). Among the ABC transporters that were highly expressed in the brain and retinal ECs relative to choroidal ECs (ABCG2, ABCB1a, ABCA5, ABCC4, and ABCC6), the expression of ABCB1a, ABCC4, and ABCC6 was significantly higher in retinal and brain EC-SP cells compared to choroidal EC-SP cells. This indicated that approximately 30% of ECs with the SP phenotype in the brain and retina express much higher levels of certain ABC transporters than choroidal stem-like EC-SP cells. Furthermore, the expression of ABCC4 and ABCC6 was significantly higher even in EC-MP cells in the retina and brain compared to choroidal EC-SP cells (Figs. 5D, 5E). The high levels of ABC transporters in the retinal and brain EC-MP cells also may contribute to maintaining the BRB or BBB. Such a specific role of ECs in the retina and brain may be the reason why the SP analysis using the Hoechst method does not work well enough to allow purification of the stem-like cells with proliferative potential from retinal and brain ECs.

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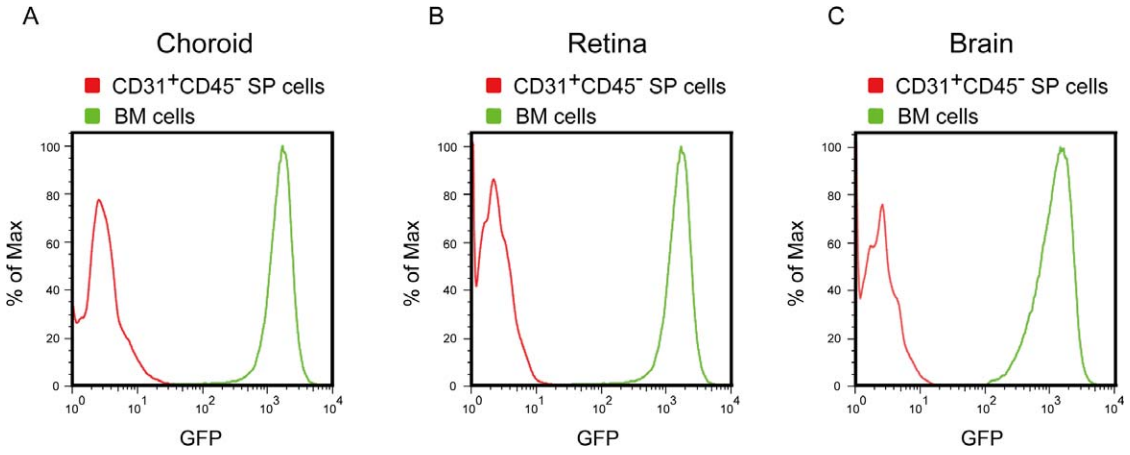

Figure 3. EC-SP cells are not derived from BM. (**A–C**) BM cells from GFP mice were transplanted into lethally-irradiated wild-type mice. At 24 weeks after transplantation, cells from the choroid (**A**), retina (**B**), and brain (**C**) were analyzed. Histogram showing GFP intensity of the $CD31^{+}CD45^{-}$ EC-SP fraction (*red*) and BM (*green*) obtained from the choroid, retina, and brain. Almost all BM cells were GFP-positive after transplantation. Note that GFP-positive $CD31^{+}CD45^{-}$ EC-SP cells were present at <0.01% of the total, suggesting no major contribution of BM cells to EC-SP cells.

EC-SP Cells in the Choroid Are Activated in Laser-Induced CNV

To study the potential of the EC-SP cells in the choroid to facilitate neovascularization in vivo, we investigated their proliferation during laser-induced CNV. A sham operation did not have any effect on the percentage of choroidal EC-SP cells (Figs. 6A, 6B). However, the percentage and absolute number of EC-SP cells in the choroid increased 6 days after laser treatment (Figs. 6C–F), while the percentage of brain and retinal EC-SP cells did not differ significantly after laser treatment (29.7 ± 2.9%, $P > 0.05$ and 32.6 ± 2.8%, $P > 0.05$ in retina and brain, respectively). These results suggested that a population of EC-SP cells in the choroid is maintained in the steady-state, but actively proliferates in response to angiogenic stimuli.

To investigate whether the EC-SP cells that had proliferated after laser treatment are BM-derived, BM cells from GFP mice were transplanted into lethally-irradiated wild-type mice, and CNV was induced by the laser 24 weeks after transplantation. No GFP-positive cells were present in the $CD31^{+}CD45^{-}$ EC-SP population even after CNV developed, indicating no major contribution of BM cells to the EC-SP cells that proliferated (Fig. 6G). Thus, EC-SP cells already present at the preexisting vessels seem to proliferate themselves in laser-induced CNV.

Discussion

In our study, we identified resident EC-SP cells in the choroidal, retinal, and brain vessels. Choroidal EC-SP cells represented 2.8% of total ECs in the choroid and had greater colony-forming potential than the majority of the EC population. The pattern of SP phenotype and colony-forming potential of the choroidal EC-SP cells was similar to the recently reported EC-SP cells isolated from limb muscle.[13] Because EC-SP cells in the limb

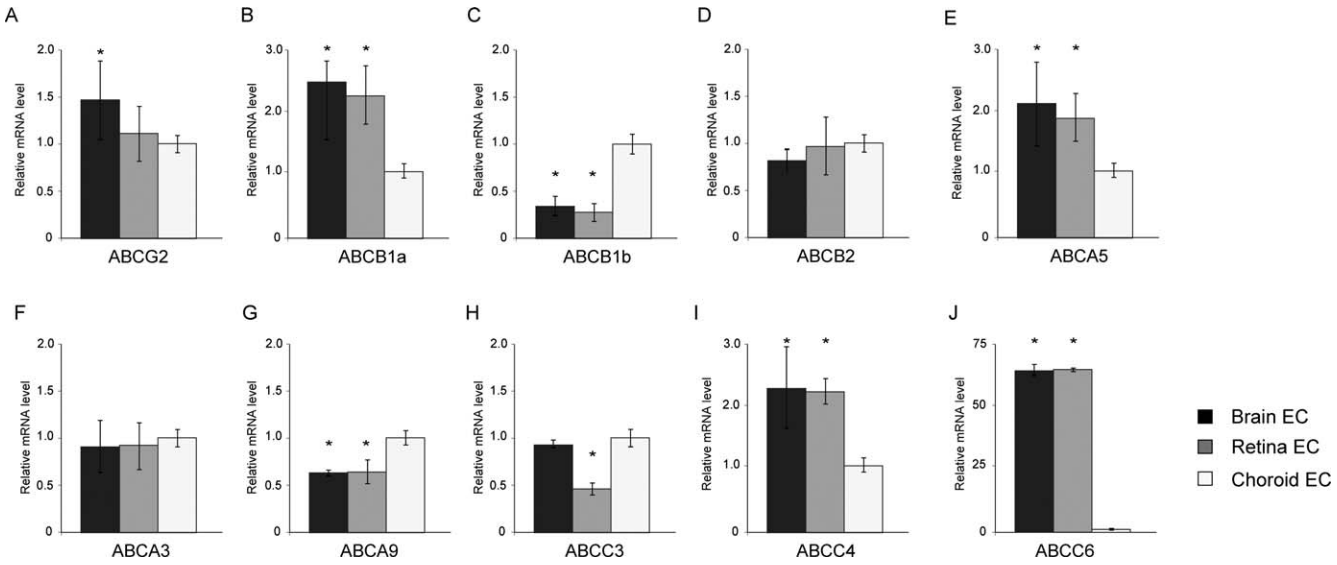

Figure 4. Quantitative RT-PCR analysis of mRNA in the choroidal, retinal, and brain EC. (**A–J**) Expression levels of ABC transporters in the choroidal, retinal, and brain $CD31^{+}CD45^{-}$ EC. Results are shown as fold-increase in comparison with choroidal ECs. Note that expression levels of ABCB1a, ABCA5, ABCC4, and ABCC6 were significantly higher in the retinal and brain ECs compared to choroidal ECs. *Error bars* are ±SEM. *$P <$ 0.05 ($n = 4$).

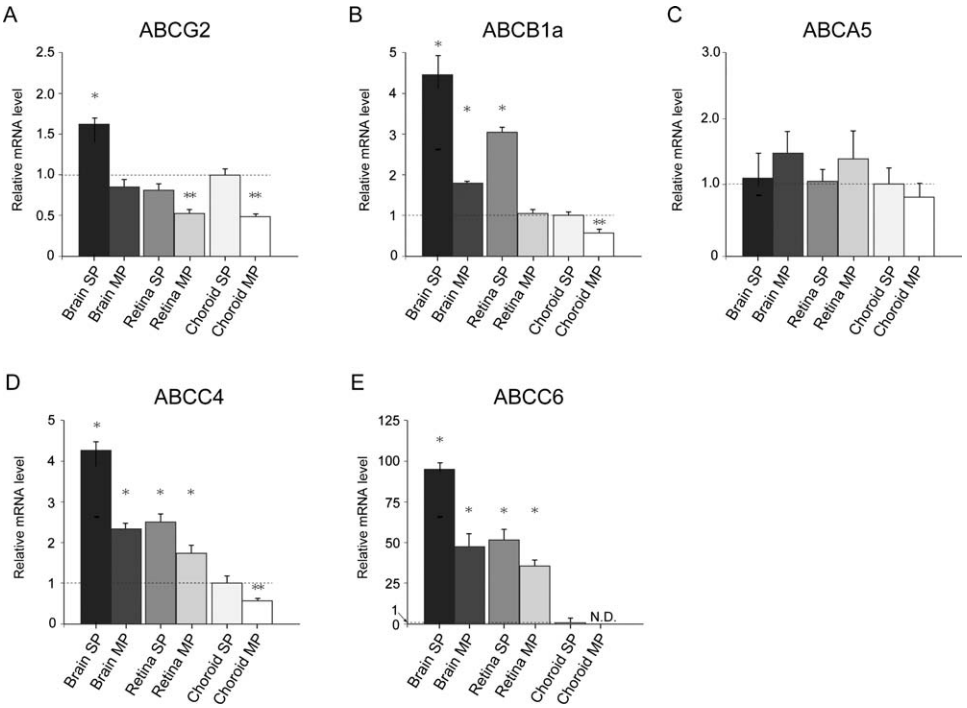

Figure 5. Quantitative RT-PCR analysis of mRNA in EC-SP and EC-MP cells from the choroid, retina, and brain. (**A–E**) Comparison of the expression levels of five ABC transporters that were highly expressed in retinal and brain ECs than choroidal ECs, as shown in Figure 4. Results are shown as fold-increase in comparison with choroidal EC-SP cells. ABCB1a, ABCC4, and ABCC6 were significantly higher in the brain and retinal EC-SP cells compared to choroidal EC-SP cells, potentially reflecting the high proportion of SP cells in the former. The ABCC4 and ABCC6 expression was significantly higher even in retinal and brain EC-MP cells compared to choroidal EC-SP cells. *Error bars* are ±SEM. Significantly higher (*$P < 0.05$) and lower (**$P < 0.05$) than choroidal EC-SP cells ($n = 3$).

muscle are regarded as colony-forming stem/progenitor-like ECs and termed "spEC" (to indicate specific ECs consisting of a hierarchical system of vascular ECs in the blood vessel), EC-SP cells from the choroid also may act as "spEC" in the choroidal vasculature.

The EC-SP cells in the choroid formed endothelial colonies positive for CD31 in culture, but did not give rise to cells other than ECs, such as hematopoietic or smooth muscle actin (SMA)–positive mural cells (data not shown). These results indicated that the EC-SP cells are lineage-committed specific ECs with high proliferative potential, which can be purified efficiently using Hoechst 33342 and flow cytometry. Although we could not investigate the in vivo contribution of EC-SP cells to choroidal angiogenesis, because of the small number of such cells that could be isolated, it is possible that EC-SP cells also have the potential to generate large number of ECs in vivo.

In contrast to the SP pattern seen in the choroid, the retinal and brain EC-SP cells contained an increased SP population, indicating that a higher proportion of ECs in those tissues can carry out ABC transporter-mediated efflux of the Hoechst dye. According to earlier reports, ABC transporters are physiologically active in retinal and brain ECs, contributing to the maintenance of barrier function and preventing cytotoxic agents from penetrating into the parenchyma.[23–25] Consistent with these reports, retinal and brain ECs showed significantly higher levels of ABCB1a (MDR1a), ABCA5, ABCC4, and ABCC6 compared to choroidal ECs in our study. Of these transporters, ABCB1a, ABCC4, and ABCC6 were expressed especially strongly in retinal and brain EC-SP cells. Therefore, we speculated that ABCB1a, ABCC4, and ABCC6 are responsible for the increase of the SP phenotype in retina and brain ECs. Because the expression levels of ABCC4 and ABCC6 were high even in retinal and brain EC-MP cells compared to choroidal EC-SP cells, EC-MP cells in the retina and brain also seemed to contribute to maintaining the BRB and BBB.

In addition to the different EC-SP pattern, EC-SP cells in the retina and brain had substantially less colony-forming potential compared to those in the choroid. Therefore, EC-SP cells in the retina and brain seemed to contain cells with as well as those without stem cell–like proliferative potential, but which still can efflux dye, leading to a lower proportion of stem cell–like cells within the EC-SP compartment. Thus, SP analysis does not work enough to purify the stem-like cells in the retinal and brain ECs as in the choroidal ECs.

Because the contribution of BM-derived EPCs to the formation of adult blood vessels in the eye and brain has been well-documented over the past decade,[7,8,26–29] we investigated whether EC-SP cells are of BM origin or not. Based on the analysis of GFP^+ BM-transplanted mice, where we saw that EC-SP cells were completely GFP-negative, we concluded that they are not BM-derived. This result is consistent with a recent report showing that the cells involved in angiogenesis are derived from local, nonhematopoietic, and noncirculating cells, according to genetic fate mapping analysis.[11] Thus, blood vessel-residing EC-SP cells may serve as a cellular source of the new ECs necessary for adult angiogenesis.

Because the choroid supplies oxygen and nourishment through its network of capillaries to the outer layers of the retina responsible for vision, it is reasonable that resident stem cell–like ECs may be present to maintain the integrity of the physiologic vasculature. However, the choroid also is associated with serious eye diseases, such as AMD and myopic degeneration.[2,3] These diseases are caused by CNV, generating pathologic new vessels in the choroid that grow beneath the retina. Because the EC-SPs have the potential to generate large numbers of ECs, we hypothesized that EC-SP cells may contribute to CNV in vivo. Using a laser-induced experimental

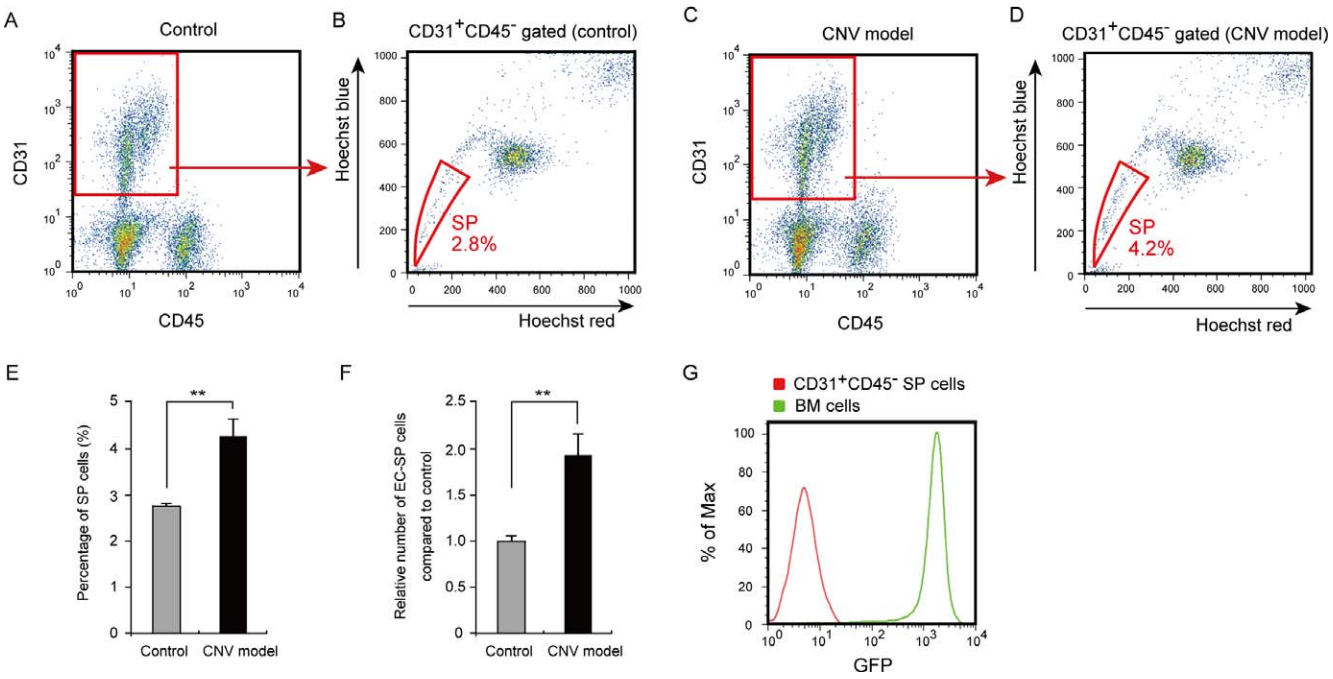

Figure 6. Choroidal EC-SP cells proliferate in laser-induced CNV. (**A–D**) Flow cytometric analysis of Hoechst 33342 staining of $CD31^+CD45^-$ ECs from the sham-operated choroid (**A**, **B**) and the choroid in which CNV had been induced (**C**, **D**). (**E**, **F**) Quantitative evaluation of the percentage (**E**) and absolute number (**F**) of EC-SP cells from 12 eyes. *Error bars* are ±SEM. $^{**}P < 0.01$ ($n = 3$, 12 eyes for each experiment, experiments repeated 3 times). (**G**) BM cells from GFP mice were transplanted into lethally-irradiated wild-type mice and, 24 weeks thereafter, CNV was induced by the laser. Cells from the choroid were analyzed 6 days after the laser treatment. The histogram shows the GFP intensity of the choroidal $CD31^+CD45^-$ EC-SP fraction (*red*) and BM (*green*). Almost all BM cells were GFP-positive after transplantation. The GFP-positive $CD31^+CD45^-$ EC-SP cells were present at <0.01% of the total, suggesting no major contribution of BM cells to EC-SP cells.

CNV model,[22] we found that the proportion and absolute number of EC-SP cells increased during CNV. The EC-SP cells that proliferated did not contain any BM-derived cells. Those results, combined with in vitro proliferative potential of EC-SP cells, indicated that EC-SP cells may possess self-renewal ability, and proliferate upon exposure to angiogenic stimuli, produce a large number of ECs, and potentially contribute to new choroidal vessels. Further studies are needed to elucidate the molecular signature of EC-SP cells, and to investigate the distribution and definitive in situ contribution of EC-SP cells during CNV formation.

Although retinal and brain vessels also are associated with angiogenesis-related diseases, such as diabetic retinopathy, cerebral infarction, and brain tumors, preexisting stem cell–like ECs must be those that contribute mainly to neovascularization. Because of the high baseline proportion, and less stem cell–like potential of EC-SP cells in the retina and brain, not only the SP phenotype, but also more specific molecular markers still are required for identifying resident stem cell–like cells and their contribution to angiogenesis. Furthermore, the physiologic role of stem cell–like cells in maintaining blood vessels remains to be investigated.

The data presented in our study suggested a potential strategy to treat blood vessel–related diseases in the future. First, in ischemic diseases, EC-SP cells may be used for proangiogenic therapy providing large numbers of vascular ECs and new blood vessels to restore blood flow in the damaged tissue. Second, in pathologic neovascularization, limiting the involvement and contribution of EC-SP cells may be useful for antiangiogenic therapy.

In summary, we identified SP cells in the $CD31^+CD45^-$ EC fraction in the eye and brain. In the choroid, EC-SP cells may represent vessel-residing endothelial stem/progenitor cells contributing to angiogenesis in vitro and in vivo. Further studies to identify the molecules responsible for the presence and proliferative potential of EC-SP cells may offer better understanding of the mechanism of angiogenesis, and development of new strategies for angiogenesis-related vascular diseases.

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