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Hematopoietic Stem Cells Provide Repair Functions after Laser-Induced Bruch's Membrane Rupture Model of Choroidal Neovascularization

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Vascular repair by adult hematopoietic stem cells (HSCs) is well-appreciated because these cells are known for their plasticity. We have shown that adult HSCs differentiate into endothelial cells and participate in both retinal and choroidal neovascularization. We asked whether HSCs participated in the wounding response by forming astrocytes, retinal pigment epithelia (RPE), macrophages, and pericytes. Lethally irradiated C57BL6/J mice were reconstituted with HSCs from mice homozygous for green fluorescent protein (GFP) and then subjected to laser-induced rupture of Bruch's membrane. After immunohistochemical examination of ocular tissue, GFP⁺ astrocytes were observed concentrated along the edge of the laser wound, where they and mural cells closely ensheathed the neovasculature. GFP⁺ vascular endothelial cells and macrophages/microglia were also evident. Large irregularly shaped GFP⁺ RPE cells constituted ~93% of RPE cells adjacent to the edge of the denuded RPE area. In regions farther away from the wound, GFP⁺ RPE cells were integrated among the GFP⁻ host RPE. Thus, postnatal HSCs can differentiate into cells expressing markers specific to astrocytes, macrophages/microglia, mural cells, or RPE. These studies suggest that HSCs could serve as a therapeutic source for long-term regeneration of injured retina and choroid in diseases such as age-related macular degeneration and retinitis pigmentosa. (*Am J Pathol* 2006, 168:1031–1044; DOI: 10.2353/ajpath.2006.050697)

studies showing that irradiated mice could be rescued from death with bone marrow transplants from a healthy donor, it became clear that HSCs could self-renew and produce all of the different cells in the blood.^{1,2} Approximately 1 of every 10,000 cells in the bone marrow is a stem cell and is defined as being Sca-1⁺, c-kit⁺, and lineage-negative (SKL), with ~1 of 10 SKL cells thought to be a true HSC that can traffic to the bone marrow and perform HSC functions.³

One main HSC function involves new blood vessel formation. Angiogenesis represents proliferation and migration of pre-existing fully differentiated endothelial cells that reside within parent vessels.⁴ The finding that bone marrow-derived cells may traffic to sites of neovascularization and differentiate is consistent with vasculogenesis,⁵ a critical paradigm for the establishment of vascular networks in the embryo. Asahara and colleagues⁶ identified the cells derived from the bone marrow capable of differentiating into neovasculature. Injection of HSCs into mice accelerates revascularization of ischemic limbs⁷ and wound healing⁸; thus, these cells are presumed to be key mediators of endothelial repair. Previously, we demonstrated that HSCs differentiate into endothelial cells and participate with resident endothelial cells to form neovascularization in the retina, choroid, and iris.^{9–11}

Stem cells are self-renewable, pluripotent cells that in adult life proliferate by a characteristic asymmetric division in which one daughter cell is committed to differentiation whereas the other remains a stem cell. These cells are also characterized by their ability to differentiate into various cell types under heterotypic environmental influ-

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The hematopoietic stem cell (HSC) is the most robust, highly characterized stem cell of the body. From the initial

ences. Plasticity refers to the ability of stem cells to acquire a mature phenotype that differs from the tissue of origin.¹² This is true for both embryonic and adult stem cells. Adult bone marrow-derived stem cells, either HSCs or mesenchymal stem cells, are multipotent and have been shown to engraft and express properties of other tissues. HSCs have been shown to transdifferentiate into cell types such as epithelium,¹³ myocardium,^{14,15} and liver.¹⁶

Several studies support the potential of neural stem cells to differentiate into ocular cells such as retinal pigment epithelial (RPE) cells when exposed to RPE-conditioned medium.¹⁷ These cells were able to attenuate the loss of photoreceptors when transplanted subretinally into Royal College of Surgeons (RCS) rats. This preservation was believed to be mediated either by the cell's ability to phagocytose the host's outer segments or by its ability to secrete soluble factors.¹⁸ To date, however, HSCs have not been shown to differentiate into RPE cells *in vitro* or *in vivo*.

Recent studies have suggested that circulating bone marrow cells can traffic to brain and transdifferentiate into neural cells. Bonilla and co-workers¹⁹ have explored the potential of adult hematopoietic bone marrow cells to differentiate into cells of oligodendroglial lineage under physiological active-myelinating conditions. They demonstrated that cells from a bone marrow subpopulation enriched in adult hematopoietic progenitor cells (c-kit⁺) express oligodendroglial-specific markers *in vivo* after intracerebral transplantation into the neonatal mouse brain. Their results suggest that adult bone marrow cells have the capacity to undergo differentiation from hematopoietic to oligodendroglial cells and support the validity of bone marrow transplantation as an alternative treatment for demyelinating diseases of the central nervous system, including multiple sclerosis.

Bone marrow cells traffic to sites of injury because they are attracted by locally produced growth factors and/or cytokines, including stromal-derived growth factor-1 (SDF-1).^{20–23} RPE cells express SDF-1 during healthy conditions and after injury.²⁴ If an SDF-1 gradient is established, cells traffic more efficiently. Conversely, if this gradient is blocked, homing efficiency is reduced to negligible levels.²⁵ Studies illustrate that HSCs and progenitor cells are sensitive to SDF-1, supporting the view that this chemokine is important for stem and progenitor cell migration in both embryonic and adult tissues.²⁶

Although vascular repair by adult HSCs is well appreciated, these cells are also known for their plasticity and can generate liver, brain, muscle, and fat.^{12,16,27–29} We

therefore asked whether circulating HSCs have the potential not only to form endothelial cells and participate in choroidal neovascularization (CNV) but also to become other cell types that participate in the development of the CNV lesion after rupture of Bruch's membrane. A CNV lesion, whether occurring clinically or after mechanical injury, typically contains numerous cell types that participate in a type of wound healing reaction in the subretinal space. We postulated that the injured subretinal space would provide cues to mobilize HSCs to this region and promote their formation into cell types other than endothelial cells, such as RPE, pericytes, smooth muscle cells, astrocytes, and macrophages.

Materials and Methods

Generation of GFP⁺ Chimeric Mice

All procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the University of Sydney Animal Ethics Committee. Reconstitution of lethally irradiated C57BL6/J mice with HSCs from GFP^{+/+} donors was performed as previously described by us.^{9,10} Induction of CNV lesions by laser rupture of Bruch's membrane was done as previously described.¹⁰

Tissue Preparation and Immunohistochemistry

Retinas were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 1 hour at 4°C. Retinal whole-mounts were prepared as described previously.³⁰ Triple-marker immunohistochemistry was used to co-visualize astrocytes (rabbit polyclonal antibody S-100 β from Chemicon, Temecula, CA), mural cells (rabbit polyclonal antibody NG2 from Chemicon), GFP⁺ cells (anti-GFP from Chemicon), vasculature (GS isolectin B4, Sigma, St. Louis, MO), and RPE (anti-RPE65, gift from J.-X. Ma [Department of Medicine, Oklahoma University, Oklahoma City, OK]; cytokeratin 8/18, clone SD3 from Novocastra, Newcastle-upon-Tyne, UK).

A maximum of three primary antibodies were applied to each specimen to determine the progeny of transplanted GFP⁺ cells in either retinal whole mounts or RPE/choroidal eyecups in treated and untreated tissue. A minimum of three specimens were analyzed per described phenomenon, and conclusions reached were representative of all specimens examined. Retinal whole mounts were permeabilized with 0.1% Triton X-100 in

Figure 1. HSCs differentiate into S-100 β ⁺ astrocytes in a rodent model of CNV. **A–C** and **D–F** show cells positive for GFP (**A** and **D**), S-100 β (**B** and **E**), and GFP, S-100 β , and GS isolectin (**C** and **F**). **A–C** show that GFP⁺ HSCs give rise to S-100 β ⁺ astrocytes with predominantly stellate morphology. Brighter GFP⁺/S-100 β ⁺ astrocytes with a simple bipolar morphology are observed interspersed among the GFP⁺ stellate astrocytes. This field of view shows the morphology of incorporated GFP⁺ astrocytes in mid-retina that are some distance from the lesion. Remarkably, these GFP⁺/S-100 β ⁺ astrocytes closely ensheath the retinal blood vessels as seen in normal retinas. These GFP⁺ astrocytes are more concentrated in the region of the laser-induced wound. Although numerous S-100 β ⁺ astrocytes are evident throughout the retina, GFP⁺/S-100 β ⁺ astrocytes are absent in the most peripheral areas of the experimental eye. **D** and **E** show a region of an experimental eye adjacent to the laser-induced wound (**arrowheads** point along margin of wound). Astrocytes in this area were observed with a predominant orientation along the wound edge. **F** shows that GS isolectin⁺ neovascularization entering the deeper layers of the mouse retina was evident at the wound edge (**arrows**). GFP⁺/S-100 β ⁺ astrocytes re-establish normal cell-cell relationships in the inner retina; however, many of the neovascular tufts (blue) in the outer retina lack close association with S-100 β ⁺ astrocytes. Representative GFP⁺/S-100 β ⁺ astrocytes are shown by **open arrowheads** in **C** and **F**.

phosphate-buffered saline (PBS) for anti-GFP and 1% Triton X-100 in PBS for S-100 β , GFAP, and GS isolectin, and samples were then blocked in 1% bovine serum albumin in PBS. Retinas were incubated overnight at 4°C with primary antibodies, washed with 0.1% Triton X-100 in PBS, incubated for 4 hours at room temperature with the appropriate secondary antibodies, and washed again. For double or triple labeling, this procedure was repeated with the different primary antibodies and appropriate secondary antibodies. Negative controls omitting a primary antibody were performed for each antibody and protocol. Vessels were labeled with biotinylated GS isolectin followed by streptavidin conjugated with Cy5 (Jackson Laboratories, Bar Harbor, ME). All antibodies and streptavidin were diluted with 1% bovine serum albumin in PBS, and washes were performed with 0.1% Triton X-100 in PBS. Retinal whole-mounts were finally mounted with the ganglion cell layer up in glycerol:PBS (2:1, v/v) or Prolong Anti-Fade (Molecular Probes, Eugene, OR).

Microscopy and Mapping

Retinal whole-mounts were examined by both deconvolution and confocal microscopy. For deconvolution fluorescence microscopy and photography, we used a Zeiss microscope (model Axioplan 2 attachment HBO 100) and Axiocam HRm camera. Confocal microscopy was performed with a Leica argon-krypton laser mounted on a Leica DMRBE epifluorescence photomicroscope. Alexa Fluor 488, Cy3, and Cy5 fluorescence was excited sequentially at 488, 550, and 649 nm, respectively. Images were processed with Adobe Photoshop V5.0 software.

Results

HSCs Generate Astrocytes

In regions at or immediately adjacent to the wound, GFP⁺ HSCs gave rise to S-100 β ⁺ astrocytes with predominantly stellate morphology (Figure 1, A, B, D, and E) as typically observed in adult mouse retina. Brighter GFP⁺/S-100 β ⁺ astrocytes with a simple bipolar morphology were observed interspersed among the GFP⁺ stellate astrocytes (Figure 1, A and B; Figure 2, A–F, M–O). GFP⁺/S-100 β ⁺ astrocytes (Figure 1, D–F) were observed in patches concentrated along the edge of the laser wound (Figure 1D, arrowheads) and were not evident in the control eye. This concentration along the wound edge is suggestive of the ability of HSCs to traffic to sites of neovascularization and exert some rescue effects. The morphology of these GFP⁺/S-100 β ⁺ astrocytes at the

edge of a wound was typically simple bipolar (Figure 1, C and F; Figure 2, A–F). Remarkably, astrocyte precursor cells also have a simple bipolar morphology during normal development,³¹ suggestive of a more immature phenotype along the astrocytic differentiation pathway.

Figure 1F shows an area of retinal neovascularization because retinal blood vessels are not normally found in these layers or with this morphology (Figure 1F, arrows). These GFP⁺ astrocytes closely ensheathed some of the vasculature in the superficial vascular plexus (Figure 1F; Figure 2, G–L), mimicking the close ensheathment of retinal blood vessels by astrocytes seen normally. On examination at higher magnification, some neovascular segments lacked a continuous astrocytic ensheathment as would be found in a normal adult mouse retina (Figure 2, D–F and J–L). Remarkably, the neovasculture in the deeper layers lacked any S-100 β ⁺ astrocytic ensheathment in this field of view (Figure 1F).

A quantitative analysis of the number of GFP⁺/S-100 β ⁺ astrocytes within 300 μ m of the lesion site showed a mean of 21.0 cells/mm² (SD = 13.1) determined over eight fields of view using a $\times 16$ objective. The numbers of GFP⁺/S-100 β ⁺ astrocytes showed significant variation with the field of view examined, where the actual counts in the eight fields of view were 18, 18, 10, 25, 51, 18, 10, and 18 cells/mm². The average number of GFP⁺/S-100 β ⁺ astrocytes farther than 300 μ m away from lesions was 1.3 (SD = 2.9, in eight fields of view at $\times 16$). The actual counts in the eight fields of view examined were 8, 0, 0, 0, 3, 0, 0, and 0 cells/mm². Although the majority of HSC-derived (GFP⁺/S-100 β ⁺) astrocytes appeared to concentrate at the wound margin, a lower number of HSC-derived astrocytes can incorporate into the existing syncytium of resident astrocytes even at distances greater than 300 μ m from the edge of the wound. As a negative control, we reacted retinas from control B57/BL6/J mice with an antibody against GFP. No GFP⁺ cells were evident throughout the entire retina. Examination of the nontreated eyes in the chimeric mouse using an antibody to GFP revealed small numbers of GFP⁺ vascular endothelial cells (VECs).

HSCs Generate Activated Retinal Microglia/Macrophages

Activated microglia/macrophages in the retina are characterized by an amoeboid morphology, enhanced expression of *Griffonia simplicifolia* (GS) isolectin B4, and a noncontact spacing pattern of distribution. Numerous GFP⁺/GS isolectin B4⁺ macrophages/microglia were evident in the experimental retina (Figure 3). These cells

Figure 2. Higher magnification views of HSCs that differentiated into astrocytes in a rodent model of CNV. Although HSC-derived astrocytes ensheathed numerous new blood vessels, astrocytic ensheathment of these new vessels was often incomplete, as shown throughout this panel. **A, D, G, J,** and **M** show GFP fluorescence. **B, E, H, K,** and **N** show S-100 β staining. **C, F, I, L,** and **O** show triple-labeling with multiple exposures showing GFP/S-100 β and GS isolectin. **A–C** show a preponderance of bipolar astrocytes aligned along the wound edge. Because bipolar astrocytes are indicative of a more immature astrocyte phenotype, this observation is supportive of HSCs giving rise to immature astrocytes after laser-induced CNV. **D–F, G–I, J–L,** and **M–O** show other examples of neovascularization after laser-induced Bruch's membrane rupture. The morphology of the vessels is not typically observed in normal mouse retina. This retinal neovasculture is closely ensheathed by GFP⁺/S-100 β ⁺ astrocytes, supportive of the role played by HSC-derived astrocytes in the wound healing process and induction of blood-retinal barrier properties. Representative GFP⁺/S-100 β ⁺ astrocytes are shown by open arrowheads in **C, F, I, L,** and **O, J:** Small numbers of GFP⁺, but not S-100 β ⁺, cells were evident. Because S-100 is only expressed by mature astrocytes, the GFP⁺ astrocyte-like cells on the left side of this field of view could represent astrocyte precursor cells before S-100 expression.

Figure 3. HSCs differentiate into VECs and macrophages/microglia in a rodent model of CNV. **A, D, G, and J** show GFP fluorescence. **B** shows S-100β staining. **C** shows triple labeling with multiple exposures showing GFP/S-100/GS-isolectin. **A–C** show isolated incorporation of GFP+ HSCs into GS isolectin+ VECs (arrowheads) and GS isolectin+ macrophages/microglia (arrow). **D–F** show isolated incorporation of GFP+ HSCs into GS isolectin+ VECs (arrowheads) and macrophages/microglia (arrows) in the superficial retinal plexus in the peripheral region of the experimental retina. **G–I** show GFP+, but weakly GS isolectin+, activated macrophages/microglia with a regular noncontact spacing distribution throughout the retinal parenchyma in the laser-injured eye. **J–L** show GFP+, but weakly GS isolectin+, vascular-associated activated macrophages/microglia in the retina of the laser-injured eye.

were regularly distributed and had an amoeboid morphology (Figure 3G).

Significant populations of GFP⁺/GS isolectin B4⁺ macrophages/microglia were evident over the entire experimental retina, with a slightly higher frequency of distribution in the periphery of the retina (Figure 3). These cells were distributed in a noncontact spacing pattern typical of macrophages/microglia. Such HSC-derived macrophages/microglia were often associated with retinal blood vessels (Figure 3, J–L). A resident population of GFP⁺/GS isolectin B4⁺ macrophages/microglia persisted in the experimental retinas (Figure 3I, arrowheads).

HSCs Generate RPE

HSCs differentiated into RPE after laser-induced Bruch's membrane rupture. Closure of the wound was incomplete at time of sacrifice 3 weeks after wounding (Figure 4A). In the area adjacent to the denuded area, RPE cells were present with significantly higher levels of GFP expression than surrounding areas (Figure 4, B–D). A small proportion of these cells had enlarged cell surface area and less regularly distributed pigment granules, resulting in irregularity to the typical hexagonal mosaic pattern. In contrast, in regions that were a substantial distance away from the wound (Figure 4E) or in the periphery (Figure 4F), fewer GFP⁺ RPE cells were present. These GFP⁺ RPE cells were smaller and more regular in appearance and formed a heterogeneous hexagonal array. HSC-derived GFP⁺ RPE cells integrated among the host RPE cells, demonstrating patches of GFP⁺ and patches of GFP[−] RPE cells adjacent to each other. These observations suggest that HSCs can differentiate into RPE and participate in wound healing in regions of laser-induced RPE damage.

As controls, posterior cups from C57BL6/J mice were subjected to GFP/GS isolectin double-label immunohistochemistry. Figure 4G shows the low level of GFP fluorescence and the regularity of the RPE mosaic in these eyes. Further, RPE cells in control C57BL6/J mice autofluoresce. RPE autofluorescence in control animals has been reported and is due to the presence of secondary inclusion bodies, such as lipofuscin, which have broad-band autofluorescence.³² In addition, the presence of other fluorophores such as retinoids, flavins, and cytochrome contribute to RPE autofluorescence. Although this level is much lower than that observed in the GFP⁺ RPE cells (Figure 4, H–J), the level of autofluorescence could be detected and captured photographically. Further, confirming earlier reports, double-nucleated RPE cells were also occasionally observed in control C57BL6/J mice (Figure 4, I and J). Double-nucleated RPE cells could be interpreted as cell fusion or phagocytosis of adjacent cells as opposed to HSC-generating RPE cells.

Two RPE-specific markers, RPE-65 and cytokeratin 8/18, were also applied to the laser-induced CNV retinas to provide further support for the conclusion that GFP⁺ HSCs generate RPE. All RPE cells were positive for RPE-65 and cytokeratin 8/18. Figure 5, A–C, shows

GFP⁺/RPE-65⁺ HSC-derived RPE cells in the region of the wound whereas Figure 5, D–F, shows GFP⁺/RPE-65⁺ HSC-derived RPE cells at the edge of the eyecup. It is unclear why the majority of GFP⁺ showed a marked increase in the expression of RPE-65. Figure 5, G–I, shows GFP⁺/cytokeratin 8/18⁺ HSC-derived RPE cells in the region of the wound. Cytokeratin bundles outline the periphery of all cuboidal RPE cells. The nuclei and larger melanin granules impart a finely vacuolated appearance to the less intensely immunopositive central cytoplasm.

We undertook a quantitative analysis of the number of GFP⁺ RPE cells in two regions: within 400 μ m of the lesion site and in a region just internal to the edge of the eyecup. Four fields of view were counted per region using the $\times 20$ objective on the Zeiss deconvolution microscope, resulting in ~ 1400 cells being counted per region. A representative field of view adjacent to the lesion is shown in Figure 4A. The average number of GFP⁺ RPE cells adjacent to the lesions was 1939 ± 488 cells/mm². The average number of GFP[−] RPE cells adjacent to the lesions was 173 ± 116 cells/mm². This equates to a mean of $92.5 \pm 3.4\%$ of RPE cells adjacent to the lesion being derived from GFP⁺ HSCs. The average number of GFP⁺ RPE cells at the edge of the eyecup was 1010 ± 189 cells/mm². The average number of GFP[−] RPE cells at the edge of the eyecup was 1801 ± 372 cells/mm². This equates to a mean of $36.2 \pm 6.8\%$ of RPE cells at the edge of the eyecup being derived from GFP⁺ HSCs. No GFP⁺ RPE cells were evident in naïve C57BL6/J mice.

Although HSC-derived GFP⁺ RPE cells concentrated at the wound margin, a significant number (36% of all RPE cells) also incorporated into the existing network of resident RPE cells at the periphery of the eyecup. This finding is consistent with our current understanding that the RPE is the site where the energy from the laser is absorbed and dissipated. Thus, it would be expected that the size of RPE wounds exceeds the actual laser spot size and suggests that laser injury stimulates HSC recruitment well beyond the region of Bruch's membrane rupture.

HSCs Generate VECs in the Retina and Choroid

A small number of GFP⁺/GS isolectin B4⁺ vascular endothelial strands were evident in the superficial vascular plexus in the experimental retina (Figure 3, A and C), suggesting the possibility of laser-stimulated recruitment into these regions. GFP⁺ VECs were observed in regions where HSC-derived astrocytes (Figure 3B) and macrophages (Figure 3, A and C) were also evident. Remarkably, GFP⁺ VECs were more frequently observed in the outer retinal vascular plexus (data not shown), as one might expect, because the outer plexus is closer to the site of laser burn. In support of this observation, widespread incorporation of GFP⁺/GS isolectin⁺ VECs was evident in the choriocapillaris (Figure 6, A–D).

Figure 5. A–C: Field of view immediately adjacent to the denuded region of the RPE double-labeled with anti-GFP and RPE-65. The denuded region is just superior to and left of the area shown. **A:** GFP⁺ RPE cells are evident throughout this field of view. **B:** All cells in this field of view are RPE-65⁺, confirming that GFP⁺ cells are RPE cells. **C:** Co-localization of GFP and RPE-65 show that the two markers co-localize to the same population. **D–F:** Field of view at the edge of the eyecup showing far fewer GFP⁺ RPE cells double-labeled with anti-GFP and RPE-65. RPE morphology of host-derived RPE cells away from the wound shows a much smaller nuclear size compared to GFP⁺ HSC-derived RPE cells. **D:** Very few GFP⁺ RPE cells are evident throughout this field of view. **E:** All cells in this field of view are RPE-65⁺, confirming that GFP⁺ cells are RPE cells. **F:** Co-localization of GFP and RPE-65 shows that the two markers co-localize to the same population. **G–I:** Field of view adjacent to the denuded region of the RPE double-labeled with anti-GFP and cytokeratin 8/18. The denuded region is just superior to and left of the area shown. Cytokeratin bundles outline the periphery of cuboidal RPE cells. The nuclei and larger melanin granules impart a finely vacuolated appearance to the less intensely immunopositive central cytoplasm. **G:** GFP⁺ RPE cells are evident throughout this field of view with a small number of GFP[−] cells evident in the lower left area of the field of view. **H:** Co-localization of GFP and cytokeratin 8/18 show that the two markers co-localize to the same population.

Figure 4. HSCs differentiate into RPE in a rodent model of CNV. **A** is a low-magnification view of the GFP fluorescence in the region surrounding a laser-induced rupture of Bruch's membrane. Closure of the laser-induced RPE wound was incomplete at 3 weeks after wounding. The **white boxes** indicate the area where the various fields of view were captured at higher magnification. **B–D** show high-magnification views of GFP⁺ RPE cells taken at various regions in the proximity of the laser-induced wound. RPE cells in these regions showed a marked variation in fluorescence intensity. Some regions are brightly fluorescent, correlating with regions of cell heterogeneity (ie, large and small cells and double-nucleated cells) while other areas have homogeneous levels of fluorescence and RPE morphology. **C** shows maximal irregularity of RPE mosaic and frequent occurrence of double-nucleated RPE in a region adjacent to the wound in the experimental eye. **D** shows a region adjacent to the wound with marked irregularity of the pigment granules. **E** shows RPE cells with a much lower level of fluorescence and more homogeneous morphology some distance away from the wound of the second experimental eye. **F** shows a higher magnification view of the RPE at the periphery in an experimental eye, with low levels of GFP⁺ fluorescence and homogenous RPE morphology. **G** shows the level of fluorescence in a C57B6/J control mouse reacted with GFP/S-100β/GS isolectin triple-label immunohistochemistry. The level of GFP fluorescence is extremely low and homogeneous. **H–J** show low- and high-magnification views showing the more regular morphology and low level of autofluorescence of the RPE in control C57B6/J mice without any immunohistochemical labeling. The RPE cell outlines are only marginally visible above background fluorescence. Double-nucleated RPE cells were also evident in this control tissue. of macrophages/microglia. Such HSC-derived macrophages/microglia were often associated with retinal blood vessels (Figure 3, J–L). A resident population of GFP[−]/GS isolectin B4⁺ macrophages/microglia persisted in the experimental retinas (Figure 3I, arrowheads).

HSCs Express Markers and Morphology of NG2⁺ Mural Cells

Examination of the posterior cups in the laser-induced Bruch's membrane rupture model showed obvious transitional zones of HSC incorporation, evident as a demarcation zone of bright and dim GFP immunofluorescence (Figure 6, E and F). HSC-derived mural cells were evident as GFP⁺/NG2⁺ somas on the outside of choroidal venules (Figure 6, G–L) and choroidal arterioles (Figure 6, M–R). The arteriole shown in Figure 6, O–R, was observed directly under a region of the choriocapillaris with large numbers of HSC-derived VECs.

Discussion

The CNV model used for these studies represents a model of acute repair and as such the HSC behavior we observed must be considered in this context. One may speculate that the stimuli for HSC recruitment in this laser-induced injury model may be more dramatic or abrupt compared to the more insidious stimuli associated with a chronic disease such as age-related macular degeneration. Thus, what we observe may represent a phenomenon associated with the laser injury conditions; however, we believe that this represents a plausible mechanism of repair throughout the retina and choroid and a possible avenue that needs to be explored therapeutically. Several caveats must be kept in mind however. For example, the transplantation approach used is one that enriches for HSCs and does not reflect normal bone marrow homeostasis. Transplantation is accomplished by using Sca-1⁺/c-kit⁺ stem cells. Although these cells are accepted as endothelial precursor cells, their plasticity is also well appreciated.

The importance of HSC plasticity has been recognized in the context of many injured tissues.^{33,34} It is well accepted that the local environment ultimately dictates the fate of the HSCs after local injury. The environment sends both attractive cues and repulsive cues to HSCs to target the cells to exactly the correct location. Stimulatory signals for attracting HSCs to the subretinal space may include changes in local growth factor expression. Cues for amplification of HSC number in the appropriate location are also active. However, the precise forces controlling the transition of these cells to their new phenotype remain elusive.

In general terms, after the initial activation of the HSCs from the bone marrow sinusoids to the circulation, the cells are not only stimulated to migrate to the area of ischemia/injury but are activated to proliferate in this region. After these initial steps HSCs are stimulated to

differentiate and finally integrate into the area forming the postlaser wound. After integration, the HSCs are expected to have proper function and participate in repair of this subretinal tissue and restore it to normal function. Not only are the cells destined for a particular cellular fate, they must be committed to the positional fate in the repaired tissue.

Ikehara and co-workers³⁵ have recently demonstrated that bone marrow stem cells can differentiate into retinal nerve cells. When these cells were injected into the vitreous cavity of untreated eyes, very few cells were found in the retina 2 weeks after injection. In contrast, when they performed laser photocoagulation before the injection of the cells, a large number of them survived 2 weeks after injection and the cells expressed neural cell-specific or retinal nerve cell-specific antigens. These cells remained viable in the retina 1 year after injection.³⁵ These same authors performed laser photocoagulation to induce CNV in GFP-chimeric mice. They corroborated our findings¹⁰ as well as those of others^{36,37} that the vascular wall cells of the CNV lesion expressed both GFP and CD31. This demonstrates that newly developed blood vessels in the CNV are derived from the bone marrow cells.

The studies presented here extend these observations by demonstrating more rigorous repair function. The incorporation of GFP⁺ cells with RPE morphology into the healing lesion has important consequences to repair of chronically injured retina and to the natural turnover or maintenance of the RPE layer. There may be areas of the RPE where the HSCs preferentially migrate into and establish contact with resident RPE because the RPE has been shown to be composed of a heterogeneous population.

The clinical significance of this study lies in the observation that HSCs can be recruited into an area of injury and can generate the unique cell populations required for repair of the injured tissue. Astrocytes contribute directly to the formation and maintenance of functional barrier properties by inducing the formation of tight junctions between VECs.³⁸ Astrocytes are intimately associated with the vasculature during development of the mammalian retina³⁹ where astrocyte migration precedes the formation of patent vessels during normal development of the human retina.^{40,41} Thus, ensheathment of the neovasculature by HSC-derived astrocytes should lead to improved barrier properties of the neovasculature after laser-induced CNV.

The main function of HSCs is one of repair because these cells are exquisitely sensitive to the cues from injured tissue throughout the body, much like white blood cells are in constant surveillance for infection within a tissue. The signal from injured tissue results in the recruitment of HSCs to these injured areas for repair, and thus

Figure 6. HSCs differentiate into choroidal VECs and choroidal mural cells in a rodent model of CNV. **A–D** show two regions where GFP⁺/GS isolectin⁺ VECs were evident in the choriocapillaries of the experimental eye. A far greater incidence of HSC-derived endothelial cells was observed in the choriocapillaries than in intraretinal vessels, perhaps as a consequence of its proximity to the RPE. **E** and **F** show two transitional zones of HSC cell incorporation in experimental choroids, evident as a demarcation zone of bright and dim GFP⁺ immunofluorescence. **G–J** show a choroidal venule with obvious GFP⁺/NG2⁺ somas on the extraluminal surface. **G–I** show the individual marker while **J** shows multiple exposures for all three markers. **K** and **L** show that GFP⁺/NG2⁺ somas of mural cells were evident on the outside of venules in the choroids. **M** and **N** show an example of GFP⁺/NG2⁺ mural cells on a choroidal arteriole. **O–R** show a second example of a choroidal arteriole with GFP⁺/NG2⁺ mural cells.

the unique plasticity of these cells speaks to their importance in repair of multiple tissues of the body. Here, we are focusing on ocular repair, which is less well studied. The phenotypic change of HSCs in the eye is quite remarkable when one considers that HSCs can express markers unique to astrocytes, RPE, mural cells, and activated microglia/macrophages.

Although the HSCs can become NG2⁺ mural cells, this represents a rare event. The limited number of GFP⁺ mural cells observed in the CNV lesion suggests that local events in the burn regulate HSC behavior and that the vessels that do form do not represent the endothelial-pericyte relationship typically observed in normal vessels.⁴² This is also the case for the astrocyte-endothelial relationship because significant lengths of the neovasculature lacked the astrocytic ensheathment, even though some of the neovasculature was ensheathed by HSC-derived astrocytes. However, this study represents a single time point, and perhaps with later analysis, more typical vascular cell interactions will be observed.

The identification of HSC-derived cells was undertaken using a combination of cell-specific markers. The GS isolectin B4 stains VECs and microglia in the rat central nervous system^{43,44} and is up-regulated on activated macrophages.^{45–47} Like von Willebrand factor, GS isolectin labels endothelial cells in culture.⁴⁸ This marker detects the smallest capillary and endothelial extensions, not always detectable by von Willebrand factor,^{41,49} and does not label pericytes or astrocytes.^{40,42,47,50} The response of the HSCs is clearly astrocytic rather than that of Müller cells; however, both cells have been intimately associated with the formation of glial vascular ensheathment in the retina.^{51,52} The phenotype of these glial cells is clearly that of astrocytes and not Müller cells.

NG2 is the rat homologue of HMW-MAA, a transmembrane chondroitin sulfate proteoglycan expressed by immature human smooth muscle cells and pericytes as well as by mature smooth muscle cells.⁵³ Nevertheless, it is expressed only sporadically and at low levels by the capillaries of quiescent vasculature.⁴² NG2 also labels a population of oligodendrocyte precursor cells in the rodent central nervous system.⁵⁴ However, because no cells of the oligodendrocytic lineage have been reported in the mouse retina or choroid, labeling of NG2⁺ cells can be confirmed as being either of pericytes or smooth muscle cells. Because only NG2 was applied to these tissues, we were unable to determine whether the GFP⁺/NG2⁺ cells were pericytes or smooth muscle cells and have instead concluded that HSCs give rise to mural cells in this rodent model of CNV.

S-100 is a dimeric protein composed of two subunits, α and β , that form three types of dimers: $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$.⁵⁵ The $\alpha\alpha$ dimer is only found in neurons⁵⁴ whereas the other S-100 proteins ($\alpha\beta$ and $\beta\beta$) are found in the cytoplasm of glial cells, particularly astrocytes. Furthermore, S-100 proteins have not been detected in microglia or oligodendrocytes in the mammalian retina.⁵⁵ In the rat retina anti-S-100 antibodies label Müller cells and astrocytes.^{56,57} However, in the rat brain anti-S-100 β antibodies label only GFAP⁺ astrocytes.^{58,59} RPE was identified

based on pigment granules, morphology, and staining with two RPE-specific markers, RPE-65⁶⁰ and cytokeratin 8/18.⁶¹

Although, some of our observations could be explained by fusion,^{62–65} we feel that this is an unlikely possibility for all of the reported results. In certain tissues fusion occurs more frequently, eg, in the injured liver and Purkinje cells in brain.⁶⁶ Bone marrow-derived cells can contribute to the regeneration of diverse adult tissues, including brain, liver, and heart, after bone marrow transplantation, and these events were initially considered to be a result of transdifferentiation of bone marrow-derived cells, supporting the emerging idea of extended plasticity of adult stem cells. However, the concept of transdifferentiation of adult stem cells has recently been called into question. Studies have now demonstrated that spontaneous cell fusion, rather than transdifferentiation, was the cause for some of the cell fate-switches of bone marrow-derived cells into hepatocytes, Purkinje cells, and cardiac myocytes *in vivo*.^{66,67}

Differentiated somatic nuclei have the flexibility to dedifferentiate when transferred into oocytes or when fused to pluripotent embryonic stem cells. Many recent publications also claim that somatic stem cells can convert into developmentally unrelated cell types both *in vivo* and *ex vivo* without such drastic cell manipulations. Some of these claims are still controversial, making it difficult for us to determine the reality of somatic stem cell plasticity. It is possible, however, that a reprogramming effect may occur in cells isolated from their physiological environment and exposed *in vitro* to stress/growth factors for prolonged periods. The potential of cell plasticity is appreciated and supported by these studies, but little is known about the mechanism.⁶⁸

The CNV lesion as initiated in this model represents a wounding model that requires repair and is thus ideally suited to evaluate HSC behavior. Although we appreciate that transdifferentiation is a dynamic process and may demonstrate temporal variation, our study evaluates this process only at one time point. Future studies may involve evaluation of a number of time points throughout the entire 3-week period after laser-induced injury. Additional studies including single cell transplantation and serial transplantations will be required to confirm true HSC plasticity. The repair involves all of the cell types that are injured, and thus it is not surprising that HSCs phenotypically change into multiple cell types that are found in this wound site.

Our data are consistent with HSCs giving rise to astrocytes. This report is the first to demonstrate this in the eye. To our knowledge this is the first report of HSCs phenotypically becoming RPE and incorporating into the RPE layer. The pattern observed in GFP⁺ HSC incorporation is consistent with wound healing in a nonproliferative cell monolayer such as the RPE or human corneal endothelium.

Retinal diseases for which HSC transdifferentiation and repair may have a role include age-related macular degeneration and hereditary retinal degenerations due to primary RPE dysfunction, such as some forms of retinitis pigmentosa. Using an individual's own HSCs would elim-

inate any immunological reaction that typically occurs with transplantation of allograft tissue.

Our results indicate the HSCs can express surface markers and morphology consistent with endothelial cells, pericytes, astrocytes, RPE, and macrophages. The GFP⁺ cells adopt the expected morphological and immunological characteristics of these cell types. This is the first study to demonstrate the successful morphological change of HSCs into these ocular cells to participate in repair of the CNV lesion. The unique plasticity of these cells may allow their manipulation for correction of ocular dysfunction in a wide variety of diseases.

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