Bone Marrow–Derived Cells Home to and Regenerate Retinal Pigment Epithelium after Injury

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PURPOSE. To determine whether hematopoietic stem and progenitor cells (HSCs/HPCs) can home to and regenerate the retinal pigment epithelium (RPE) after induced injury.

METHODS. Enriched HSCs/HPCs from green fluorescent protein (gfp) transgenic mice were transplanted into irradiated recipient mice to track bone marrow–derived cells. Physical damage was induced by breaching Bruch’s membrane and inducing vascular endothelial growth factor A (VEGFA) expression to promote neovascularization. RPE damage was also induced by sodium iodate injection (40 mg/kg) into wild-type or albino C57Bl/6 mice. Cell morphology, gfp expression, the presence of the Y chromosome, and the presence of melanosomes were used to determine whether the injured RPE was being repaired by the donor bone marrow.

RESULTS. Injury to the RPE recruits HSC/HPC–derived cells to incorporate into the RPE layer and differentiate into an RPE phenotype. A portion of the HSCs/HPCs adopt RPE morphology, express melanosomes, and integrate into the RPE without cell fusion.

CONCLUSIONS. HSCs/HPCs can migrate to the RPE layer after physical or chemical injury and regenerate a portion of the damaged cell layer. (Invest Ophthalmol Vis Sci. 2006;47:2108–2113) DOI:10.1167/iovs.05-0928

RPE dysfunction has been linked to many devastating eye disorders, including age-related macular degeneration,1 and to hereditary disorders, such as Stargardt disease and retinitis pigmentosa.2 Attempts to repair the RPE include transplantation of RPE cells into the subretinal space. Animal studies, RPE transplantation in humans, and macular relocation surgery have all shown that replacing diseased RPE with healthier RPE can rescue photoreceptors, prevent further visual loss, and even promote visual improvement.3,4 Also, recent work on human RPE patch graft transplantation demonstrates survival and rescue of photoreceptors for a substantial time after grafting and holds some promise. Rescue of RPE and photoreceptors beyond the area of donor cell distribution suggests that diffusible factors are also involved in the repair process. However, some problems exist, including the ability to obtain an adequate source of autologous RPE and that homologous cells have been associated with rejection. Fetal or adult transplanted RPE cells attach to Bruch’s membrane with poor efficiency and do not proliferate.5 These transplantation procedures are complex, associated with high complication rates, and often result in only short-term success.

RPE integrity is an essential component for retinal function and visual health. The RPE consists of a monolayer of cuboidal cells that separates the photoreceptors and the choroid.6 The RPE is an integral component of the visual process that removes rod and cone outer segment fragments by phagocytosis when they are shed into the intraretinal space. This process is critical to the uptake, processing, transport, and release of retinol (vitamin A) and retinoids. The RPE is also the barrier between the highly vascular choroid and the photoreceptors of the neural retina, forming part of the blood-retinal barrier through its tight junctions.7 The RPE is responsible for the movement of ions and water for the maintenance of a proper state of dehydration for visual clarity, and its pigmentation absorbs stray light that would otherwise degrade the visual image.8,9 Stem cell plasticity refers to the ability of stem cells to acquire a phenotype that differs from their tissue of origin.10 Adult stem cells of the bone marrow include hematopoietic stem cells (HSCs), which are multipotent and have been shown to transdifferentiate into multiple tissues such as endoderm,11,12 epithelium,9,10,11 myocardium,12 and liver.13,14 These cells represent a renewable source of cells within our bodies and harnessing the regenerative ability of the HSCs may aid in the cure of degenerative diseases. In our model, the bone marrow transplantation was performed using CD117 (c-kit) cells. This technique enriches for HSCs and hematopoietic progenitor cells (HPCs).15

In addition to HSC/HPC transdifferentiation, the ability for the HSCs/HPCs to home to an area of injury is a quintessential characteristic. When injury occurs, cytokines and chemokines are released into the blood, causing an inflammatory response. HSCs/HPCs home along this chemokine gradient to repair areas of injury. Stromal cell–derived factor 1 (SDF-1) has been shown to be the primary cytokine for HSC/SPC mobilization.16–18 SDF-1 has also been shown to be upregulated in damaged tissues thus facilitating recruitment of stem-progenitor cells to promote repair.19 The RPE is known to constitutively express and to secrete SDF-1 to maintain the choroid.20 This SDF-1 expression modulates HPC engraftment of the choroid.21

In this article we examined two models of injury. Our first model consisted of physical damage to the RPE layer by needle rupture of Bruch’s membrane and then injection of recombinant adenovirus associated virus vascular endothelial growth factor A (AAV-VEGFA) into the subretinal space. This leads to choroidal neovascularization.9 We found that this damage also induced cytokine and chemokine expression, allowing circulating HSCs/HPCs to incorporate into the RPE layer. To further classify this phenomenon of HSCs/HPCs regenerating RPE, we looked at RPE damage induction by sodium iodate. From previous literature, injection of sodium iodate results in RPE cell growth inhibition, which is consistent with our results.22

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destruction and blood-retinal barrier dysfunction in a patchy pattern. The current viewpoint is that once the RPE is terminally differentiated, it does not renew itself by cell division. In this study, we have shown that HSCs/HPCs regenerate RPE after acute injury. Donor HSCs/HPCs take on aspects of RPE morphology when examined by confocal and transmission electron microscopy (TEM). These cells produce melanin and are clearly seen as pigmented RPE-like cells when the recipient mouse is an albino. Finally, these cells are not a result of fusion of GFP+ cells with host RPE because, fluorescence in situ hybridization detects the presence of both the Y and X chromosomes in these GFP+ RPE cells from female recipients of male HSCs/HPCs.

METHODS

All animal procedures were reviewed and approved by the University of Florida Animal Care and Use Committee, performed in an Association for Assessment of Laboratory Animal Care (AALAC)-approved facility and treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Bone Marrow Transplantation

Bone marrow was flushed from the long bones of male gfp+ STOCK Tg(GFP)5Nagy/J mice (The Jackson Laboratory, Bar Harbor, ME). The bone marrow cells were further enriched for HSCs/HPCs by magnetic activated cell sorting, using a ckit+ or CD117+ microbeads (Miltenyi MACS, Auburn, CA). CD117+/gfp+/male cells (2500) were transplanted into lethally irradiated (9.5 Gy from a Cs137 source) female HSCs/HPCs.

Mechanical Rupture of Bruch’s Membrane with rAAV-VEGFa Injection-Induced Choroidal Neovascularization and RPE Damage

C57Bl/6 mice (The Jackson Laboratory), 8 to 10 weeks old (n = 7) were transplanted with 2500 CD117+/gfp+/male cells and allowed to recover for 3 months. This is a standard bone marrow transplant that effectively replaces the female recipient hematopoietic system with the male gfp+ hematopoietic system. This method enables easy detection of HSC/HPC-derived progeny in any tissue. Next, Bruch’s membrane was physically ruptured, and rAAV-VEGFa was injected subretinally. One month after virus injection, the mice were anesthetized and perfused with 4% paraformaldehyde (PFA) via cardiac puncture. After perfusion, the eyes were enucleated and placed once again in 4% PFA for 30 minutes and then in PBS for 30 minutes. The neural retina was dissected from the posterior cup (RPE-choroid-sclera complex). The posterior cup was flat-mounted with four to seven radial cuts and mounted (Vectashield; Vector Laboratories, Burlington, CA) before observation using a microscope (Olympus IX70; Olympus America Inc., Melville, NY) coupled to the microscope system software (Bio-Rad Confocal 1024 ES; Bio-Rad, Hercules, CA).

Chemical Injury with Sodium Iodate

C57Bl/6 mice, 8 to 10 weeks old (n = 5 per time period), were injected with 40 mg/kg of sodium iodate (Sigma-Aldrich, St. Louis, MO) in the retro-orbital sinus. RPE injury was monitored over a 14-day period by immunohistochemistry. Mice were euthanatized at days 0, 1, 3, 7, and 14. The eyes were enucleated and placed in 4% PFA for 1 hour at 4°C. Eyes were washed in PBS, embedded in paraffin, cut in 5-μm sections, placed on slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA), and used later for immunohistochemistry.

Albino Mouse Model

Sodium iodate injections (40 mg/kg) were administered 4 days before a bone marrow transplant. Bone marrow cells (2500) enriched for CD117+ from male STOCK Tg(GFP)5Nagy/J bone marrow were transplanted into lethally irradiated (9.5 Gy) female C57Bl/6/Tyr+/-/J (n = 8) mice (Jackson Laboratory). Approximately 30 days after transplantation, animals were anesthetized and killed and their posterior eye cups analyzed for the presence of pigment within the RPE layer. BM engraftment levels of animals were analyzed via flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) for CD3, CD11b, and B220 (BD PharMingen, San Diego, CA) to observe the T cell, macrophage, and B-cell bone marrow populations, respectively.

Eyes were immediately enucleated and fixed in 4% PFA for 1 hour, placed in PBS overnight. The posterior cup was flat mounted before examination for pigmented cells. Bright-field microscopy was used to analyze the presence of melanosomes within the RPE cells (BX51; Olympus).

Transmission Electron Microscopy

Eyes were enucleated and fixed for 1 hour in cold 4% PFA-1% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4). Specimens were washed four times for 15 minutes in 0.1 M cacodylate buffer containing 0.1 M glycine. Eyes were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a methanol series to propylene oxide, and infused and embedded in epoxy resin.

Ultrathin sections on nickel grids were oxidized in 1% (wt/vol) aqueous periodic acid in at 250-W power (PELCO BioWave Microwave; PELCO, Ted Pella, Inc., Irvine, CA), 30°C, with a cycle of 2 minutes on, 2 minutes off, and 2 minutes on. Grids were washed in phosphate-buffered saline (PBS; pH 7.4) four times for 1 minute followed by blocking with 4% (vol/vol) cold-water fish gelatin in PBS at 250 W and 30°C, with a cycle of 2 minutes on, 2 minutes off, and 2 minutes on. Grids were reacted with polycrystalline rabbit anti-GFP antibody (Chemicon International, Temecula, CA) at 250-W power and 30°C, with a cycle of 2 minutes on, 2 minutes off, and 2 minutes on, followed by washing twice for 1 minute with PBS and twice for 1 minute with Tris-buffered saline (TBS). Grids were incubated with goat anti-rabbit IgG conjugated to 12 nm colloidal gold (Jackson Immunoresearch, West Grove, PA) at 250 W and 30°C, with a cycle of 2 minutes on, 2 minutes off, and 2 minutes on. Grids were then rinsed with TBS twice for 1 minute, followed by rinsing three times for 1 minute with deionized water, followed by poststaining for 2 minutes with 2% (wt/vol) aqueous uranyl acetate. Controls were omission of the primary antibody and use of secondary antibody alone. Grids were examined and photographed in a transmission electron microscope (1200EX; JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.

Immunohistochemistry

Paraffin-embedded 5-μm sections from 4% PFA-fixed, enucleated mouse eyes were deparaffinized and rehydrated. Slides were treated for 25 minutes (Target Retrieval Solution; DakoCytomation, Carpinteria, CA), rinsed in Tris-buffered saline, and blocked in normal horse serum (Vector Laboratories) for 20 minutes at room temperature. Sections were incubated overnight at 4°C in a combination of 1:50 polyclonal goat anti-SDF-1α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:50 polyclonal rabbit anti-mouse HIF-1α (Novus Biologicals, Littleton, CO). Isotype and concentration of the goat and rabbit IgG was matched with goat and rabbit IgG from Vector Laboratories. The secondary antibodies, AlexaFluor donkey anti-rabbit 488 nm and AlexaFluor donkey anti-goat 594 nm (Invitrogen, Eugene, OR), were applied 1:200 for 1 hour at room temperature in the dark. After extensive washes in Tris-buffered saline, the slides were counterstained and mounted in antifade medium (Vectashield; Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was documented via a laser scanning spectral confocal microscope (TCS SP2; Leica Microsystems Heidelberg GmbH, Wetzlar, Germany). Fluorescence in situ hybridization (FISH) probes were acquired from Cambio (Dry Drayton, Cambridge, UK) for the X (FTC) and Y (Cy3) chromosomes from their mouse chromosome paint product line (StarFISH; Dry Drayton). Slides containing deparaffinized 5-μm sec
Incorporation of HSCs/HPCs into Damaged RPE after Bruch’s Membrane Rupture

Choroidal neovascularization was induced by puncturing Bruch’s membrane and injecting rAAV-VEGFα subretinally in mice \( (n = 7) \) engrafted with \( gfp^- \) HSCs/HPCs.\(^2\) We observed \( gfp^- \) cells contributing to the RPE layer. These cells had RPE morphology by confocal microscopy. Figure 1 shows posterior cups of transplant-recipient mice that underwent choroidal neovascularization and physical injury of the RPE. The \( gfp^- \) cells were found only at the sites of injury. The donor-derived cells were cuboidal in shape, the hallmark morphology of RPE. However, the tightly packed RPE layer and the heavy pigmentation of the layer make conclusive morphologic analysis by light microscopy difficult. Therefore, we sought additional injury models in which the RPE layer is damaged, to confirm these initial observations.

Assimilation of Donor HSCs/HPCs with Host RPE after Sodium Iodate Injury

Sodium iodate has been shown to damage the RPE.\(^2\) The level of RPE damage can also be controlled by the dose of sodium iodate administered. We chose the dose of 40 mg/kg for our experiments.

Sodium iodate is known as an RPE toxin, but the effects on hematopoiesis have not been addressed. Two groups were compared to determine whether sodium iodate had any effect on \( gfp^- \) cells incorporation into the RPE layer. Figure 2A displays a photomontage of a representative retinal flatmount of a mouse that underwent a bone marrow transplant before injection of sodium iodate. There was little to no incorporation of \( gfp^- \) HSCs/HPCs in the RPE layer. We also observed a decrease in marrow cellularity after sodium iodate indicative of hematopoietic damage (data not shown).

Damage exerted by sodium iodate on the RPE results in a patchy pattern on fluorescein angiography.\(^2\) In Figure 2B we observed this patchy appearance of \( gfp^- \) HSCs/HPCs within the RPE layer when mice underwent bone marrow transplantation 4 days after sodium iodate. By administering sodium iodate before bone marrow transplantation, we achieved a greater degree of donor-derived HSC/HPC incorporation within the damaged RPE layer. Once again, confocal analysis suggests that many of the donor-derived cells had adopted the cuboidal morphology that is characteristic of RPE.

Production of Pigmented RPE by Donor HSCs/HPCs in Sodium Iodate–Treated Albino Mice

The presence of pigment and the tightly associated nature of RPE make it difficult to use light microscopy to confirm that donor-derived HSCs/HPCs are producing the hallmark melanosomes of RPE. Therefore, we used C57BL/6J-Tyr^r-/-J female mice, which are albino and produce no melanosomes, and transplanted enriched CD117^-/gfp^-/male HSCs/HPCs into them 4 days after sodium iodate treatment. This approach enables the easy identification of melanosome-producing cells of donor origin as an indicator of HSC/HPC regeneration of the damaged RPE layer. It is important to note that there is no difference in RPE necrosis between pigmented and nonpigmented mouse strains and that the amount of damage is solely dependent on the amount of sodium iodate injected.\(^2\) Figure 3 shows the presence of pigmented cells in the RPE layer using bright-field microscopy. Figures 3A and 3C are retinal flatmounts of eyes that were damaged by needle puncture in a model of choroidal neovascularization and physical injury of the RPE. The cups of transplant-recipient mice that underwent choroidal morphology by confocal microscopy. Figure 1 shows posterior cup flatmounts of animals treated with sodium iodate after (A) and before (B) bone marrow transplantation. (A) Posterior cup flatmount 1-month after sodium iodate injection of mice previously receiving transplants of enriched \( gfp^- \) BM. There was little to no contribution of \( gfp^- \) cells. (B) Posterior cup flatmount of a mouse treated with sodium iodate 4 days before \( gfp^- \) bone marrow transplantation showing an increase in \( gfp^- \) cells. Scale bar, 200 μm.
mounts of normal C57Bl/6j and C57Bl/6J-Tyr<sup>-/-</sup>/J mice RPE, respectively. Figure 3B shows a cluster of approximately 10 pigmented cells in our albino mice transplanted with STOCK Tg(GFPU)5Nagy/J enriched bone marrow. Melanosomes can only be derived from the donor HSCs/HPCs in which the pigment allele is present.

To overcome the limitations of bright-field microscopy for the morphologic identification of RPE pigmentation, we confirmed the presence of pigmented, gfp<sup>+</sup>, donor-derived cells with RPE morphology within the damaged RPE layer by TEM. Anti-gfp antibodies marked donor-derived cells with gold particles. We observed immunogold (gfp<sup>+</sup>) cells with full RPE morphology by TEM within the RPE layer depicted in Figures 4A and 4B. The arrows mark clusters of gfp<sup>+</sup> protein and the asterisks the melanosomes, indicating that the RPE cells are derived from the gfp<sup>+</sup> HSCs/HPCs.

**Diploidy of HSC/HPC-Derived RPE**

Bone marrow cells have been shown to fuse spontaneously with embryonic stem cells in vitro in the presence of IL-3. Subsequent studies have demonstrated that cell fusion can also occur in vivo for a limited number of cells. To determine whether cell fusion is responsible for the gfp<sup>+</sup> RPE cells in the RPE layer, we performed sex mismatched transplants in the C57Bl/6J-Tyr<sup>-/-</sup>/J and C57Bl/6J-Tyr<sup>-/-</sup>/J mouse. Notice the cuboidal shape and pigment, which can only be derived from the transplanted HSCs/HPCs (B). Scale bar, 30 μm.

**HSC/HPC Recruitment to the Damaged Retina**

We examined the presence of hypoxia-inducible factor (HIF)-1 and SDF-1 protein in mice exposed to sodium iodate over the 14-day time period. HIF-1 is a transcription factor that initiates a cascade of events including inducing expression of SDF-1, a stem cell chemoattractant. We have previously shown that induction of SDF-1 is critical for HSC derived neovascularization in damaged retinas. Figure 6 shows that HIF-1 and SDF-1 are expressed in the uninjured retina and in the retina after sodium iodate–induced damage.

**FISH of X and Y chromosomes of donor-derived RPE.** Retinal cross-sections of mice subjected to the sodium iodate damage model, were analyzed for both an X (green) and a Y (red) chromosome. That these cells were donor derived is indicated by the presence of the X and Y chromosomes (filled triangles). Circulating lymphocytes (open triangles) were derived from the donor BM as well, but did not incorporate into the RPE layer. Scale bar, 40 μm.

![Figure 3. Pigmented RPE cells in albino injury model.](image-url) Albino mice received transplants of wild-type bone marrow after undergoing the sodium iodate injury model for RPE damage. (A) The RPE layer in a normal C57Bl/6j mouse. (B) Several RPE cells in the RPE layer of an C57Bl/6J-Tyr<sup>-/-</sup>/J (albino) mouse transplanted with STOCK Tg(GFPU)5Nagy/J-enriched HSCs/HPCs. (C) The RPE layer of a normal C57Bl/6J-Tyr<sup>-/-</sup>/J (albino). Notice the cuboidal shape and pigment, which can only be derived from the transplanted HSCs/HPCs (B). Scale bar, 30 μm.

![Figure 4. EM of gfp protein in RPE.](image-url) (A, B) Transmission electron micrographs of cells in the RPE layer with RPE morphology and immunogold staining of gfp protein (arrows). GFP localized only in the retinal pigment epithelium (RPE). There was no localization in the neural retina or endothelial cells of the neural retina. BM, Bruch’s membrane; N, nucleus; RPE, retinal pigment epithelium; CC, choroid; (●) melanosome.

![Figure 5. FISH of X and Y chromosomes of donor-derived RPE.](image-url) Retinal cross-sections of mice subjected to the sodium iodate damage model, were analyzed for both an X (green) and a Y (red) chromosome. That these cells were donor derived is indicated by the presence of the X and Y chromosomes (filled triangles). Circulating lymphocytes (open triangles) were derived from the donor BM as well, but did not incorporate into the RPE layer. Scale bar, 40 μm.
(Fig. 6C) showed a decrease in HIF-1 and SDF-1 from normal control levels. Days 3 (Fig. 6D), 7 (Fig. 6E), and 14 (Fig. 6F) after sodium iodate depict the expression of HIF-1 and SDF-1 as the RPE damage progressed. These data show that HIF-1 remained cytoplasmic, suggesting that HIF-1 and the ischemia-induced cascade is not activated by sodium iodate damage. SDF-1 is the key recruitment factor for HSCs/HPCs and it was expressed on the apical and basal side of the RPE. SDF-1 did not appear to increase its expression within the RPE layer during sodium iodate injury. This finding may account for the relatively low level of HSC/HPC incorporation within the RPE.

**DISCUSSION**

In this study, bone marrow–derived cells regenerated RPE in two different acute injury models. HSC/HPC-derived cells adopted an RPE morphology containing the hallmark melanosomes (experiments with albino mice receiving transplanted wild-type HSCs/HPCs analyzed by TEM), without any evidence of cell fusion (FISH for X and Y chromosomes). These data suggest that RPE-like cells can be derived from HSCs/HPCs in vivo in response to injury.

The ionizing radiation used to myeloablate the recipient animals may have induced additional damage in the RPE layer. The effects of ionizing irradiation on the RPE have not been described. ERG recordings from animals exposed to irradiation compared with mice fitted with head protection composed of lead were used to see whether whole-body irradiation affects retinal function. We saw no significant decrease in b-wave amplitude in irradiated mice compared with protected controls (Harris and Scott, unpublished data, 2004). Therefore, we doubt that damage from ionizing irradiation is a factor in our observed results.

The RPE is very similar in function to the monocye of the blood in phagocytosis and antigen expression such as CD14 and CD68. We speculate that the RPE could be another example of a terminally differentiated tissue-specific monocyte-macrophage.

Sodium iodate is a well known RPE toxin, but the effects on hematopoiesis have not been analyzed. Our data suggest that there is a significant decrease of gfp cell contribution when the BM transplantation occurs before sodium iodate administration. We postulate that the sodium iodate, which damages RPE cells, damages the hematopoietic lineage(s) that regenerates the RPE as well. Therefore, administering sodium iodate 4 days before BM transplantation allowed the toxin to clear the systemic circulation, thus minimizing injury to the transplanted donor HSCs/HPCs and increasing their contribution to repopulating the host bone marrow and regenerating RPE.

Approximately 0.1% of RPE cells were HSCs/HPCs derived after sodium iodate injury of albino mice that received transplants of pigmented HSCs/HPCs. This low number may reflect the approach we used to induce injury. If this is the case, other types of injury is needed, to generate higher proportions of HSCs/HPCs-derived RPE. The 40-mg/kg dose of sodium iodate used in our study may have been too low to induce significant HSC/HPC recruitment. SDF-1 was present, but not induced further during the injury model. Unfortunately, higher doses of sodium iodate cause significant damage to the retinal architecture. The resident RPE microenvironment may no longer be able to provide the developmental cues to induce HSC/HPC differentiation into RPE. The importance of SDF-1 in the regeneration of a wide variety of injury models suggests that artificially increasing the levels of SDF-1 would induce more HSCs/HPCs to the site, which may augment regeneration.

Caution should also be exercised in extrapolating the results from acute injury models of RPE regeneration to chronic diseases such as age-related macular degeneration. In this study, HSCs/HPCs regenerated cells in the RPE layer in response to acute injury. From our analysis, the HSC/HPC-derived cells exhibited the phenotype characteristic of RPE, but only additional long-term studies with more functional data will say for sure.

**References**


