Facile isolation and the characterization of human retinal stem cells

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This study identifies and characterizes retinal stem cells (RSCs) in early postnatal to seventh-decade human eyes. Different subregions of human eyes were dissociated and cultured by using a clonal sphere-forming assay. The stem cells were derived only from the pars plicata and pars plana of the retinal ciliary margin, at a frequency of ≈1:500. To test for long-term self-renewal, both the sphere assay and monolayer passaging were used. By using the single sphere passaging assay, primary spheres were dissociated and replated, and individual spheres demonstrated 100% self-renewal, with single spheres giving rise to one or more new spheres in each subsequent passage. The clonal retinal spheres were plated under differentiation conditions to assay the differentiation potential of their progeny. The spheres were produced all of the different retinal cell types, demonstrating multipotentiality. Therefore, the human eye contains a small population of cells (≈10,000 cells per eye) that have retinal stem-cell characteristics (proliferation, self-renewal, and multipotentiality). To test the *in vivo* potential of the stem cells and their progeny, we transplanted dissociated human retinal sphere cells, containing both stem cells and progenitors, into the eyes of postnatal day 1 NOD/SCID mice and embryonic chick eyes. The progeny of the RSCs were able to survive, migrate, integrate, and differentiate into the neural retina, especially as photoreceptors. Their facile isolation, integration, and differentiation suggest that human RSCs eventually may be valuable in treating human retinal diseases.

The ciliary margin of the mouse eye contains retinal stem cells (RSCs) (1) that have the capacity to proliferate *in vitro*, with the potential to make all of the cell types of the neural retina (1, 2). Stem cells are defined as cells with the capacity for long-term self-renewal and the ability to make the cell types of their tissue of origin (multipotentiality). In contrast, progenitor cells have a limited proliferative capacity and tend to be unipotential. There is evidence that the human eye also may contain RSCs (1), and we hypothesized that their most likely niche would be in the pigmented or nonpigmented epithelial layer of the ciliary margin at the peripheral edge of the retina. In the mouse eye, each sphere arises from a single pigmented cell, suggesting that rare pigmented cells in the ciliary margin are undifferentiated stem cells (1). Certainly, most pigmented cells in the spheres are retinal pigmented epithelium (RPE) progenitors. The human neural retina has been shown to contain retinal progenitor cells (3) similar to the retinal progenitor cells isolated from the mouse eye (4), lending credence to the idea that the human adult eye may contain earlier lineage RSCs. In addition, the human brain contains neural stem cells (NSCs) located in homologous positions to those found in the mouse brain (5–10), but these human brain NSCs have slower proliferation kinetics than the mouse brain NSC *in vivo* (11, 12) and *in vitro* (5, 6); the human NSCs require 3 weeks to produce a clonal neurosphere colony compared with only 1 week for the mouse. We show here that human RSCs are as readily isolated and grown as mouse RSCs and indeed proliferate much faster in culture than human brainstem cells. Thus, there is no ubiquitous species difference between mouse and human stem cells and indeed there is a remarkable conservation of mouse and human RSC properties, which bodes well for the eventual therapeutic potential of human RSCs.

Materials and Methods

***In Vitro* Assay for Stem Cells.** Human eyes were procured from the Eye Bank of Canada (Toronto, ON) or the Eye Bank of the Jules Gonin Eye Hospital (Lausanne, Switzerland) within 24 h postmortem. The eyes were dissected in artificial cerebral spinal fluid (1). The eye was cut in quarters and the eye was divided up into five different areas, including the posterior iris, pars plicata, pars plana, RPE, and the neural retina (see Fig. 5*A*, which is published as supporting information on the PNAS web site). The dissociated cells were counted and plated at either 10 or 20 cells per μl in serum-free media (SFM) (1) containing either no exogenous growth factors epidermal growth factor (EGF), fibroblast growth factor 2 plus heparin (F+H), or EGF plus fibroblast growth factor plus heparin (E+F+H) (Sigma). Cells proliferated to form floating sphere colonies in cell suspension, and these retinal spheres were counted after 7 days in culture. (For detailed methodology, see *Supporting Methods*, which is published as supporting information on the PNAS web site).

Clonality. To test the clonality of the cells that are capable of forming spheres, cells from primary retinal spheres were plated at a density of 1 cell per well in individual 96-well plates in the presence of F+H. Wells were visually inspected 2 and 24 h after plating to ensure that only single cells were in each well. After 7 days in culture, the wells were inspected for the presence or absence of spheres.

Self-Renewal of Stem Cells. Individual clonal human retinal spheres were passaged by using either the sphere suspension assay or the monolayer culture assay.

Sphere Suspension Passaging. Single RSC sphere colonies were dissociated as described in ref. 1 (see also *Supporting Methods*). Single spheres were passaged every 7 days to test self-renewal capacity of the RSCs.

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Abbreviations: RSC, retinal stem cell; NSC, neural stem cell; RPE, retinal pigmented epithelium; EGFP, enhanced GFP; EGF, epidermal growth factor; F+H, fibroblast growth factor 2 plus heparin; E+F+H, EGF plus fibroblast growth factor plus heparin; MAP2, microtubule-associated protein 2.

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Monolayer Passaging. Primary human ciliary epithelial cells were cultured at 10 or 20 cells per μl for 7 days in SFM containing EGF, F+H, or E+F+H. Individual retinal spheres then were transferred to a 24-well plate containing 10% FBS (GIBCO) and the same growth factors that were used in primary retinal sphere formation, leading to monolayer cultures. After 3 days in the presence of FBS, the sphere became adherent to the bottom of the well and cells began to migrate out from the sphere (see Fig. 3*M*). For passaging, cells were trypsinized (0.05% trypsin–EDTA, Sigma) and plated in six-well plates (at a density of 10 cells per μl) and then in 75-cm^2 flasks (at a density of 30 cells per μl). Every week, the cells were either expanded or induced to differentiate.

Differentiation and Immunostaining. To assay the differentiation potential of the RSC-derived colonies, individual clonally derived spheres were plated as whole spheres or dissociated spheres (10 cells per μl) in 24-well plates on a substrate of 50 ng/ml laminin, poly-L-ornithine plus laminin, or EHS Natrix (BD Biosciences) in the presence of 1% FBS/10 ng/ml fibroblast growth factor 2/2 μg/ml heparin or 20 ng/ml EGF/10% FBS. Media were changed every 3–4 days, and cells were allowed to migrate and differentiate over the course of 3 weeks, at which time the plates were fixed in fresh 4% paraformaldehyde. Immunohistochemical analysis of the cells was tested by using antibodies directed to specific markers. Nestin, Pax6, and Chx10 were used for undifferentiated cells; βIII tubulin, microtubule-associated protein 2 (MAP2), and neurofilament-M were used for retinal ganglion cells; Rho4D2, Rom1, 309L, and D2P4 were used for photoreceptor cells; 10E4, glial fibrillary acidic protein, vimentin clone V9, and human-specific nestin were used for Müller glia; HPC-1 and Pax6 were used for amacrine cells; calbindin was used for horizontal cells and off-bipolar cells; Chx10 and protein kinase C-α were used for rod bipolar cells; RPE65 and human-specific bestrophin were used for RPE cells; desmin and cytokeratin were used for smooth muscle cells; O4 was used for oligodendrocytes; and clone 5B5 was used for fibroblasts. The secondary antibodies were tetramethylrhodamine B isothiocyanate goat anti-mouse IgG, tetramethylrhodamine B isothiocyanate goat anti-Mouse IgM, FITC goat anti-rabbit, Alexa Fluor 568 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit. After immunostaining, cells were counterstained with Hoechst 33258 nuclear stain. (A list of the antibodies and their suppliers is published in supplementary information online). Staining was visualized by using an Olympus inverted fluorescent microscope, Roper HQ camera, and Olympus MICROSUITE imaging software. Confocal images were taken with a Zeiss LSM410 system.

Transplantation. ***Lentivirus construct.*** Replication-defective, self-inactivating lentiviral vectors containing an internal ribosome entry site sequence followed by enhanced GFP (EGFP) were prepared (refs. 13 and 14 and *Supporting Methods*).

Lentiviral transfection of stem cells. Human adult RSC spheres were dissociated, and the cells were seeded at 1.0×10^5 cells per well in 1 ml of SFM. Cells were transfected at an multiplicity of infection of 100 for 24 h at 37°C in 5% CO_2. After infection, the cells were harvested, washed twice, plated at 10 cells per μl in SFM containing F+H, and allowed to proliferate for 7 days. The spheres that arose were visualized with a fluorescent microscope, and only green spheres were used for the transplantation experiment.

Transplantation into the mouse eye. The green fluorescent human eye spheres were dissociated into single cells and resuspended in Hanks' balanced salt solution at a concentration of 20,000 cells per μl. Approximately 10,000 cells in 0.5 μl were transplanted into the vitreous cavity of postnatal day 1, immune-deficient NOD/SCID host mice (15–17) ($n = 16$) that had been anaesthetized by using the inhalant halothane. A small puncture was made in the sclera to facilitate the insertion of the pulled microcapillary pipet containing the cell suspension. The tip of the pipette was visualized through the cornea to ensure delivery of the cells into the vitreous. Subretinal injections were not used for these studies because when dissociated or intact retinal stem cell spheres are transplanted into the subretinal space, they do not integrate into the host eye (B.L.K.C., personal observation). Cells were injected by using a modified mouth pipette with barriers to ensure that we did not contaminate the cells or the host mouse. The eyelids were then pulled back together and awake animals were put back with their dams. Mice were killed 28 days later, their eyes removed and fixed in 4% PFA. Eyes were sectioned at 14 μm on a Bright cryostat. The eye sections were then visualized under a fluorescent microscope to detect the human enhanced GFP cells.

Transplantation into the chick eye. Human retinal progenitor cells (passage no. 5) were labeled with the PKH26 Red Fluorescent Cell Linker kit (Sigma) for general cell membrane labeling . A pulled microcapillary needle was used to inject ≈10,000 cells into the vitreous of an embryonic day 3.5–4 chick eye. Eyes were collected at different time points, fixed in 4% formaldehyde for 1–6 h, washed in PBS for at least 1 h, and put in 30% sucrose until the eye sank. Eyes were embedded in Tissue-Tek (Fisher Scientific), and 20-μm-thick sections were cut for further processing.

Results

Isolation of Proliferative Retinal Cells. To assay for RSCs, human eyes from early postnatal ages to the seventh decade were dissected so that each area of the anterior eye [iris, pars plicata, pars plana, RPE, and the anterior neural retina (Fig. 5*A*)] were plated and cultured in SFM for a minimum of 7 days (1). Human pars plana and pars plicata contained cells that could proliferate over the course of 7 days to form spheres that contained both undifferentiated pigmented and nonpigmented cells (Fig. 5*B*), ranging in size from 100 μm to 400 μm in diameter. Note the round and smooth shape of the spheres (Fig. 5*C*). When pars plana and plicata cultures were kept for 30 days, the number of spheres did not increase significantly. The numbers of spheres that were derived from the ciliary margin areas (pars plana and pars plicata) were somewhat variable between individuals. However, the similar frequency of stem cells at the young postnatal ages and over the next 6 decades suggests that the RSC population is maintained from birth to old age (Fig. 1*A*). The variability in sphere-forming frequency may be due to the variable postmortem times before culturing or to other variables that are uncontrolled for when using human tissue.

The ciliary margin of the human eye contains RSCs that show similar growth factor responsiveness *in vitro* to mouse RSCs. The pars plana and plicata both gave rise to clonal spheres (see below) *in vitro* without the presence of exogenous growth factors, and these spheres contained both pigmented and nonpigmented cells (Fig. 1*B*). However, with addition of growth factors (either F+H or E+F+H) both ciliary margin areas gave rise to significantly more spheres compared with conditions without exogenous growth factors ($P < 0.05$). The pars plicata gave rise to significantly more retinal spheres than the pars plana ($P < 0.05$) (Fig. 1*B*), indicating that the frequency of stem cells located in the pars plicata is higher than the pars plana.

The posterior iris (adjacent to the pars plicata) also contained colony-forming cells that could give rise to spheres. The iris spheres contained both pigmented and nonpigmented cells. The spheres that arose from the iris only came from the posterior half of the iris and only proliferated in the presence of exogenous growth factors. The neural retina, RPE, and the anterior half of the iris, did not give rise to spheres, even when grown *in vitro* for 30 days. To test whether the proliferation kinetics of some of the human RSCs were similar to the human brain NSCs, which can take 3 weeks or longer to form spheres in culture (5, 6, 10, 11),

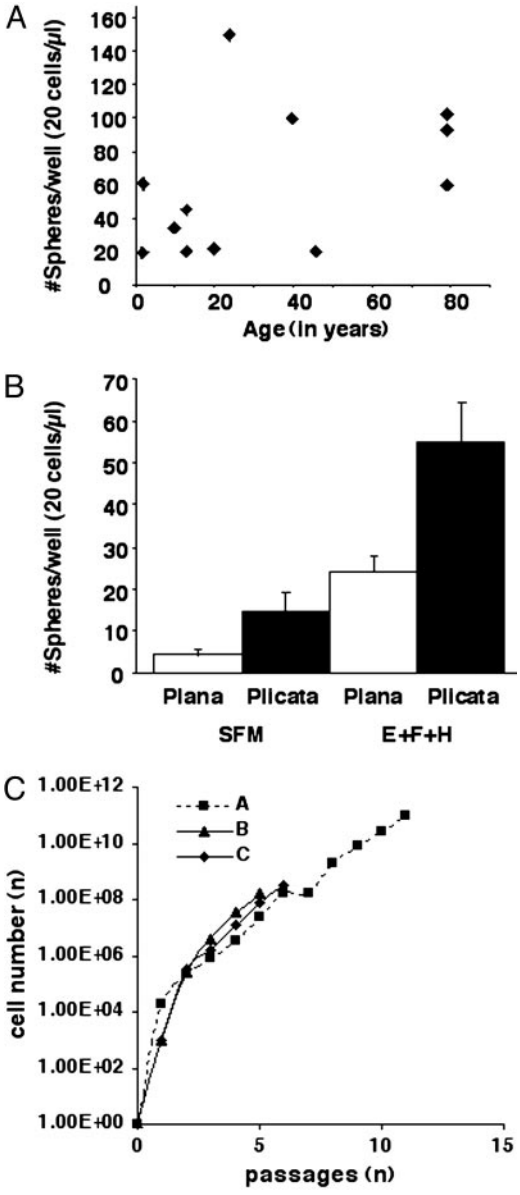

Fig. 1. Primary sphere formation and self-renewal. (*A*) Retinal sphere numbers appear to be grossly similar across postnatal ages in humans when the dissociated cells of the ciliary margin are plated at 20 cells per μl. (*B*) Ciliary margin cells proliferate in 7 days to form clonal spheres that can arise without the presence of exogenous growth factors, but growth factors significantly increase the number of cells capable of forming a clonal sphere *in vitro*. (*C*) The spheres are capable of long-term self-renewal as demonstrated by monolayer passaging of single clonal spheres that arose from a single stem cell. Each line represents the expansion of a RSC from three different donor eyes.

some human retinal cells were kept in culture for at least 30 days, but no new spheres formed after the initial 7-day culture period. Because the human sphere-forming retinal cells were able to proliferate and form spheres within 7 days, similar to mouse RSC derived spheres (1), human RSCs appear to be fundamentally different from the human NSCs found in the brain.

To demonstrate unequivocally the clonal origin of the retinal spheres, single primary cells from the pars plicata were plated in each well of 96-well plates to test whether cells from the ciliary margin of the human eye were capable of proliferation to form clonally derived spheres. In the 1,012 wells that contained only single cells on day 1, a total of four spheres arose after 7 days. The presence of these spheres indicates that there is a subpopulation of single pigmented cells in the ciliary margin that is capable of forming clonal spheres at a calculated frequency of $\approx$1:253. In the primary cultures that were plated at a density of 20 cells per μl, a comparable frequency of stem cells from the ciliary margin (1:500) was observed. Thus, the single-cell-per-well experiment suggests that the vast majority of spheres that arose from 20-cells-per-μl wells also were clonally derived.

Self-Renewal. Because long-term self-renewal is a key property that distinguishes stem cells from short-lived progenitor cells, we used *in vitro* passaging to test the self-renewal properties of individual spheres. Single spheres from the pars plana, plicata, and the iris were dissociated into single cells, replated in the presence of growth factors, and cultured for 7 days to test their ability to form secondary spheres (indicating that the cell that formed the primary sphere underwent asymmetric or symmetric divisions to self-renew itself). Initially, each primary stem cell from the pars plana and plicata gave rise to a large clonal expansion in the numbers of stem cells within each single primary sphere (average no. of secondary spheres from each primary sphere = 91.2 $\pm$ 9.6), with 100% of all single spheres giving rise to at least one new sphere. However, over subsequent passages up to passage no. 13 (the longest sphere passaging attempted), the numbers of new spheres that formed from dissociation of each single sphere dropped significantly by the third passage and leveled off at a ratio of just greater than one sphere giving rise to one sphere (passages 3–13 = 1.3 $\pm$ 0.6). This finding suggests that the RSC that initiated the primary sphere underwent many symmetric divisions when forming a primary sphere to produce a significant number of stem cells (as assayed by the number of secondary spheres from a single dissociated primary sphere). In subsequent passages, the number of new spheres dropped dramatically to one sphere giving rise to one or two new spheres, indicating that the stem cell was now undergoing primarily asymmetric divisions but was still self-renewing. In contrast, iris spheres were unable to form any secondary spheres, suggesting that they are progenitor cells that retain some limited proliferative capacity.

Monolayer Passaging. To reveal the expansion potential of RSCs, we used the monolayer culture. By using this technique, we were able to generate 10^{11} cells in $<$2 months (passaging every 7–10 days) in the presence of EGF (n = 6) (Fig. 1*C*) or F+H (n = 2) from one single retinal sphere (clonally derived from a single cell). This expansion corresponds to a 37-cell doubling capacity, the limit being not yet determined. These cells were passaged 11 times and are continuing to passage as monolayers. Once the monolayer culture is established, if FBS is removed from the culture, a few cells from the monolayer are then able to proliferate to form retinal spheres that appear identical to the primary spheres derived from the ciliary margin, indicating that there are still RSCs present in these cultures. The proliferating monolayer cells remain in a retinal precursor state, as indicated by localization of nestin expression (89%; n = 4) (Fig. 2*N*). Thus, RSCs may have a broader expansion potential than can be demonstrated by using the single-sphere passaging assay.

Multipotentiality. The RSCs from human eyes were tested for their differentiation potential. Single spheres from both pars plana and pars plicata were cultured in differentiation media and the cells immunohistochemically stained for the different cell types of the neural retina and the RPE. The RSC spheres contain progeny that were capable of giving rise to the different cell types of the neural retina and the RPE (Fig. 2 *A–L*). The retinal spheres contained variable ratios of each differentiated cell type. A relatively large percentage of the cells that migrated from the RSC spheres remained undifferentiated even after 3 weeks

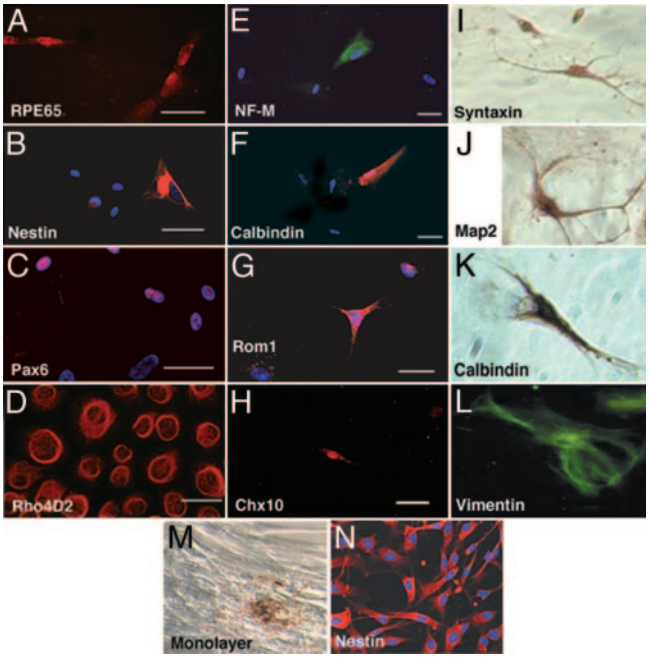

Fig. 2. Multipotentiality. The progeny of single spheres formed clonally from human RSCs differentiate to form all of the cell types of the retina when plated on adherent substrates. The nuclei of all cells are stained with the blue dye Hoechst 33328 in *B*, *C*, and *E–G*. (*A–H*) These cell types include RPE cells (RPE65$^+$) (*A*), undifferentiated cells (nestin$^+$) (*B*), retinal progenitors/amacrine [Pax6$^+$] (*C*), photoreceptor cells (Rho4D2$^+$) (*D*), retinal ganglion cells (neurofilament-M$^+$) (*E*), horizontal cell (calbindin$^+$) (*F*), photoreceptor cells (Rom1$^+$) (*G*), and bipolar cell (Chx10$^+$) (*H*). (Scale bars: 20 μm.) (*I–L*) Monolayer culture differentiation. In the presence of 2% FBS and EGF, the cells differentiated into neural cells. Several neuronal markers were observed: syntaxin (*I*), MAP2 (*J*), and calbindin (*K*). (*L*) These cells were also capable of differentiating along a glial lineage as indicated by vimentin staining when plated in the presence of EGF and 10% FBS. (Magnification: *A* and *B*, ×200; *C–I*, ×400.) (*M*) Upon addition of 10% FBS, the clonal sphere cells spread out of the retinal sphere (remnant of sphere indicated by an arrow), leading to a monolayer culture. (*N*) Monolayer culture stained with nestin (red).

Table 1. The percentage of cell types after 21 days in differentiation culture

Cell type	Antibody	Percent positive	Hoechst cells, *n*
Amacrine	HPC-1*	2.5 ± 1.6	374
	Pax6$^+$/nestin$^-$	8.8 ± 2.0	286
Horizontal/off-bipolar cells	Calbindin	12.2 ± 9.7	241
Rod photoreceptor	Rho4D2	34.5 ± 9.1	643
	309L	24.6 ± 10.7	201
Rod outer disc protein	Rom1	8.5 ± 4.6	134
Müller glia/astrocytes	GFAP	19.7 ± 10.6	267
RPE	RPE65	0.6 ± 0.8	754
Pigmented cells	None	7.0 ± 1.7	265
Undiff. neural and Müller glia	Nestin	34.5 ± 6.1	629
RPCs and amacrine	Pax6	68.5 ± 15.1	423
RPCs and rod bipolar cells	Chx10	40.4 ± 9.7	410
Rod bipolar cells	Chx10$^+$/nestin$^-$	2.9 ± 0.8	236

Human RSC spheres are multipotential. Plated spheres were immunostained with markers for all of the cell types of the neural retina and RPE and for undifferentiated cells. The percent positive cells column indicates the average number of positive cells (± SEM) in each well compared with the number of nuclei stained with Hoechst. The total numbers of Hoechst cells are >100% because some of the markers mark overlapping cell populations. Nestin, Pax6, and Chx10 mark retinal progenitors, and Pax6 and Chx10 mark retinal progenitors and differentiated cell populations (amacrine and bipolar cells, respectively). Rho4D2 and 309L label rhodopsin in photoreceptors, and Rom1 labels an outer segment disc protein in photoreceptors. Undiff., undifferentiated; RPC, retinal progenitor cells; GFAP, glial fibrillary acidic protein; none, no antibody used.

*HPC-1 also is found in progenitor cells that can make either amacrine or horizontal cells but is only found in postmitotic amacrine cells in mature retina.

(Table 1), but the majority of the differentiated cells were photoreceptor cells, as indicated by the presence of Rom1, Rho4D2, and 309L staining. Other cell types of the neural retina also were present at lower frequencies (Table 1), including amacrine and horizontal cells (HPC1 and calbindin), RPE (RPE65), rod bipolar cells (Chx10 and protein kinase C), Müller glia (10E4, glial fibrillary acidic protein, vimentin, nestin), and retinal ganglion cells (βIII tubulin, neurofilament-M). Retinal ganglion cells differentiated preferentially in low-density cultures (assayed by plating dissociated spheres or in wells where cells have migrated away from the majority of the other cells). Many neural retinal cell types appeared to differentiate in patches, which may indicate that differentiation of these cell types may be influenced by cell–cell contact; indeed, there is some evidence that cell density can play a role in biasing retinal cells down different cell lineages or at least in the survival of these lineages (18). The photoreceptors (indicated by photoreceptor-specific antibodies) were single-rounded cells in culture and did not look morphologically like photoreceptors, consistent with photoreceptors requiring the presence of an intact RPE to form their characteristic shape and outer discs (19). In addition, the cells that stain with photoreceptor markers did not stain with any other retinal cell antibodies, nor did any of the cultured progeny of RSCs stain with antibodies to oligodendrocytes, fibroblast, or smooth muscle antigens. The results suggest that photoreceptors are differentiating in the RSC cultures, but may be missing the proper signals to form their *in vivo* morphologies.

***In Vivo* Transplantation.** *In vivo* repopulation represents the most stringent test for stem cell function. To evaluate the *in vivo* regulation potential of RSCs, we used a transplantation assay. To visualize human RSCs and their progeny once they were transplanted into the host mouse eye, we used a lentiviral construct with an EGFP reporter. The lentiviral construct containing EGFP was transduced into RSCs derived from the human ciliary margin *in vitro*, as demonstrated by the presence of some wholly fluorescent green spheres that arose 7 days after single cells were infected (Fig. 3*A Inset*). Not all of the RSCs were transduced with EGFP as demonstrated by the presence of nonfluorescent spheres and spheres containing only a small number of green cells (presumably because a later progenitor in the sphere was infected). Therefore, under a fluorescent microscope, only spheres expressing the GFP ubiquitously were selected for transplantation.

To give the human retinal cells an optimal host environment, cells were injected into the mouse retina when endogenous developmental factors induce neuronal differentiation. The dissociated EGFP$^+$ RSCs and their progeny were transplanted into the eyes of postnatal day 1, immune-deficient NOD/SCID mice, at a time when photoreceptors and Müller glia are being maximally induced to differentiate in the host eye. The NOD/SCID system has been used extensively to assay hematopoietic stem cells and represents and ideal host for repopulation studies (15). The developing mouse host eye provides a permissive environment for donor cells to integrate into the eye in the presence of proliferative and differentiation signals from the host eye. In addition, the developing eye lacks a mature glial limitans that has been shown to inhibit transplanted donor cells from migrating into the neural retina (20). Of the 16 mice that

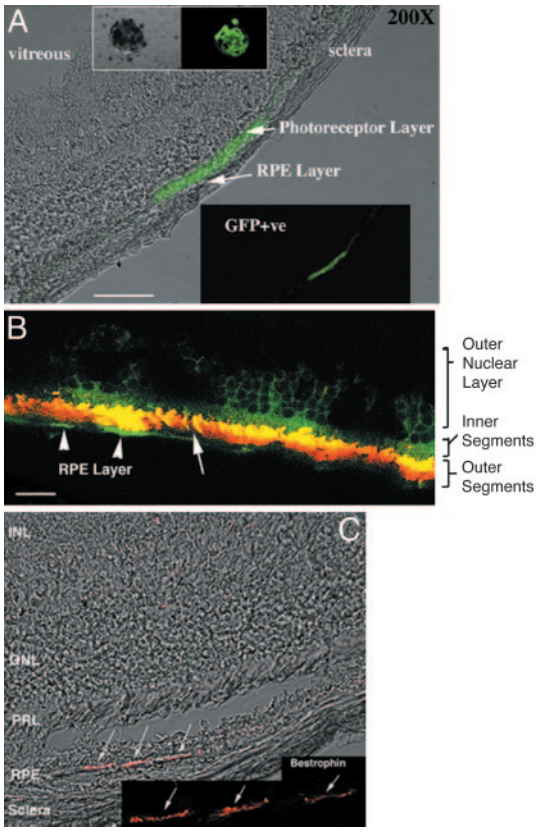

Fig. 3. Transplantation into the mouse eye. (*A*) The EGFP$^+$ human RSCs and progeny are able to survive and integrate into the neural retina and the RPE. The green fluorescence is primarily located in the photoreceptor inner and outer segments. (Scale bar: 250 μm.) (*Insets*) The same human RSC sphere (210-μm diameter) derived from an EGFP–lentiviral-labeled stem cell in phase contrast and under GFP fluorescence (GFP+ve). The pigmentation of some of the cells in the sphere masks the fluorescence of the cells, making the ubiquitously green-labeled sphere appear mottled. (*B*) The EGFP$^+$ human RSCs and progeny (green) are capable of making Rom1$^+$ (red) photoreceptor cells. The long arrow points to some isolated Rom1$^+$ outer segments of the photoreceptor layer double-labeled with EGFP (yellow). The arrowheads indicate EGFP$^+$/Rom1$^-$ cells located in the RPE layer. (Scale bar: 50 μm.) (*C*) Transplanted human RSCs and progeny that differentiated as RPE cells labeled with a human-specific RPE marker (bestrophin). (*Inset*) A magnified fluorescence image of the bestrophin staining in the RPE layer. The bestrophin$^+$ cells are EGFP$^-$, indicating that the lentivirus has been silenced in these human donor cells. INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer.

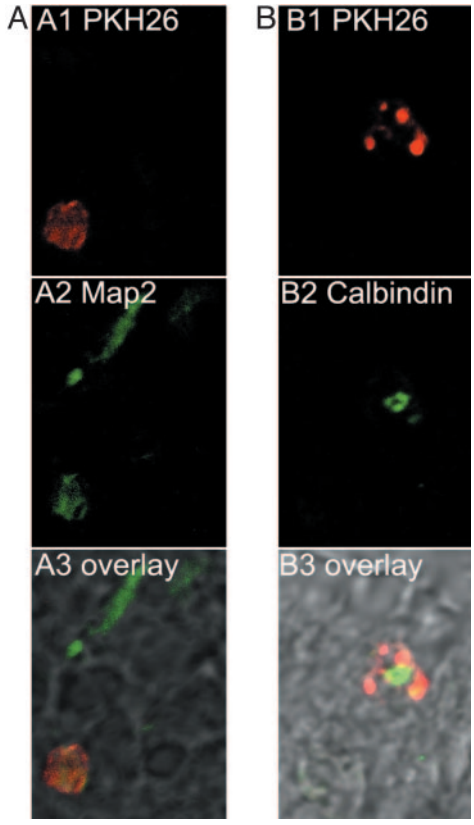

Fig. 4. Transplantation into the chick eye. Human cells labeled with the cell membrane dye PKH26 (red) were injected into the vitreous of the eye and collected at different time points. (*A*) Labeled human cells were injected into the vitreous at embryonic day 3.5 and collected at embryonic day 12. (*Top*) A human cell incorporated into the ganglion cell layer (GCL) of the retina (red). (*Middle*) This cell also expressed the RGC marker MAP2 (green). (*Bottom*) The differential interference contrast overlay. (*B*) Labeled human cells were injected at embryonic day 4 and collected at embryonic day 10. A human cell (*Top*, red) expressing the horizontal cell marker, calbindin (*Middle*, green) was also detected near the ganglion cell layer of the developing chick retina. (*Bottom*) The differential interference contrast overlay.

had EGFP-expressing human retinal sphere cell transplants, 12 mice had green fluorescent cells present after the 28-day survival period. In eight of the mice that had EGFP$^+$ cells, there was morphological integration and migration of the human RSC sphere progeny into the distal outer layer of the neural retina and the RPE (Fig. 3). The green fluorescence is localized to the photoreceptor inner and outer segments (Fig. 3*A*), which contain the majority of the photoreceptor cell cytoplasm. In most cases, the cells that integrated appeared to have migrated from the vitreous through the neural retina to reach the photoreceptor layer and the RPE. It is also possible that some of these cells may have migrated along the needle track, and into the subretinal space between the RPE and the photoreceptor layer. However, in a few cases EGFP$^+$ cells were found on the opposite side of the eye from the injection site, indicating that these cells must have integrated into these outer retinal cell layers by migrating through the developing neural retina rather than along the injection needle tract. Photoreceptor-specific immunostaining of the host eye sections demonstrated that EGFP$^+$ human retinal sphere cells had integrated into the photoreceptor layer and were expressing Rom1, a rod photoreceptor outer segment protein (Fig. 3*B*). The many fewer EGFP$^+$ cells that integrated into inner retinal layers did not express photoreceptor markers. A few were Müller glia, but the majority of the EGFP$^+$ inner nuclear layer cells were of an undetermined type. Interestingly, there are some cells that are EGFP$^-$ that express either a human-specific RPE marker (bestrophin) (Fig. 3*C*) or more infrequently, a human-specific nestin, which marks Müller glial cells (Fig. 6, which is published as supporting information on the PNAS web site). (Antibody controls are shown in Fig. 7, which is published as supporting information on the PNAS web site.) These findings indicate that silencing of the lentivirus occurs in some of the differentiating donor cells. In the other four mice in which EGFP$^+$ cells were found after 28 days, the cells appeared to have stayed near the injection site and appeared to cause an immune reaction, as indicated by host photoreceptor rosettes forming in the host neural retina.

To reveal whether the human RSCs can also generate the earlier-born neurons formed during embryonic retinogenesis, we injected expanded human RSCs labeled with the PKH26 dye (21) into the vitreous of developing chick eyes, at embryonic day 3.5. We observed that in the four eyes injected, groups of cells

migrated into the ganglion cell layer and the inner nuclear layer. Eight days after the transplantation, we labeled the cells with different antibodies and scored only cells having strong dye labeling and a specific neuronal marker. In all of the eyes injected ($n = 4$), we observed that human retinal progenitor cells adopted retinal cell fates, as demonstrated by the expression of MAP2 antigen in retinal ganglion cells (Fig. 4*A*) or the calbindin antigen labeling in horizontal cells (Fig. 4*B*). These experiments demonstrate that the human RSCs, even after sustained expansion, can generate cells able to respond to environmental cues occurring during different developmental eye stages. The data also suggest that there may be extrinsic, developmental cues biasing the fate of the transplanted cells at different developmental ages. These experiments also show the remarkable conservation among chick, mice, and human of developmental mechanisms.

Discussion

These studies represent a characterization of RSCs in human eyes. RSCs were present from birth until the seventh decade (further evidence of the long-term self-renewal of RSCs). Human RSCs possess properties similar to mouse RSCs, including their rapid proliferation kinetics *in vitro* and their ability to proliferate without the addition of exogenous growth factors. This feature suggests that the phenotype of RSCs is highly conserved across mammals. These results further imply that the human RSC is fundamentally different from the human brain NSC, because human adult forebrain stem cells proliferate more slowly and require exogenous growth factors to proliferate (unlike the human RSCs) (5, 6).

In the mammalian eye, photoreceptors comprise ≈70% of the total retinal cell population (22). Consistent with these *in vivo* results, when the clonal human RSC spheres are plated in culture, the majority of the differentiated cells express mature photoreceptor markers. However, in culture, the human RSC-derived photoreceptors displayed only a rounded morphology with no or few processes. Nevertheless, after transplantation of the human RSCs and their progeny into the early host eyes, RSC-derived cells take on a photoreceptor morphology and appropriately express a major rod photoreceptor protein, Rom1, in the outer segment of the cells. Moreover, the human cells were able to integrate into different neuronal layers at appropriate developmental times. Thus, ganglion cells were formed from RSCs injected in early development, whereas photoreceptors were formed from RSCs transplanted later in development. In the future, similar experiments will need to be done in adult mouse hosts with retinal degeneration phenotypes; further experiments will also need to identify the extrinsic factors biasing the cells toward specific cell fates to examine the potential of this work for human therapy.

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1. Tropepe, V., Coles, B. L. K., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R., van der Kooy, D. (2000) *Science* **287,** 2032–2036.
2. Ahmad, I., Tang, L. & Pham, H. (2000) *Biochem. Biophy. Res. Commun.* **270,** 517–521.
3. Yang, P., Seiler, M. J., Aramant, R. B. & Whittemore, S. R. (2002) *Exp. Neurol.* **177,** 326–331.
4. Jensen, A. M. & Raff, M. C. (1997) *Dev. Biol.* **188,** 267–279.
5. Vescovi, A. L., Gritti, A., Galli, R. & Parati, E. A. (1999) *J. Neurotrauma* **16,** 689–693.
6. Roy, N. S., Benraiss, A., Wang, S., Fraser, R. A., Goodman, R., Couldwell, W. T., Nedergaard, M., Kawaguchi, A., Okano, H. & Goldman, S. A. (2000) *J. Neurosci. Res.* **59,** 321–331.
7. Carpenter, M. K., Cui, X., Hu, Z. Y., Jackson, J., Sherman, S., Seiger, A. & Wahlberg, L. U. (1999) *Exp. Neurol.* **158,** 265–278.
8. Vescovi, A. L., Parati, E. A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Shoellere, P., Cova, L., Arcellana-Panlilio, M., Colombo, A. & Galli, R. (1999) *Exp. Neurol.* **156,** 71–83.
9. Arsenijevic, Y., Villemure, J.-G., Brunet, J.-F., Bloch, J. J., Deglon, N., Kostic, C., Zurn, A. & Aebischer, P. (2001) *Exp. Neurol.* **170,** 48–62.
10. Kukekov, V., Laywell, E., Suslov, O., Davies, K., Sheffler, B., Thomas, B., O'Brien, T., Kusakabe, M. & Steindler, D. (1995) *Exp. Neurol.* **156,** 333–344.
11. Sanai, N., Tramontin, A. D., Quinones-Hinojosa, A., Barbaro, N. M., Gupta, N., Kumwar, S., Lawton, M. T., McDermott, M. W., Parsa, A. T., Manuel-Garcia Verdugo, J., *et al*. (2004) *Nature* **427,** 740–744.
12. Rakic, P. (2004) *Nature* **427,** 685–686.
13. Miyoshi, H., Blowmer, U., Gage, F. G. & Verma, I. M. (1998) *J. Virol.* **72,** 8150–8157.
14. Tahara-Hanaoka, S., Sudo, K., Ema, H., Miyoshi, H. & Nakauchi, H. (2002) *Exp. Hematol.* **30,** 11–17.
15. Dick, J. E., Bhatia, M., Gan, O., Kapp, U. & Wang, J. C. (1997) *Stem Cells* **15,** 199–207.
16. Young, M. J., Ray, J., Whiteley, S. J., Klassen, H. & Gage, F. H. (2000) *Mol. Cell Neurosci.* **16,** 197–205.
17. Chacko, D. M., Rogers, J. A., Turner, J. E. & Ahmad, I. (2000) *Biochem. Biophys. Res. Commun.* **268,** 842–846.
18. Ezeonu, I., Wang, M., Kumar, R. & Dutt, K. (2003) *DNA Cell Biol.* **22,** 607–620.
19. Caffe, A. R., Visser, H., Jansen, H. G. & Sanyal, S. (1989) *Curr. Eye Res.* **8,** 1083–1092.
20. Kinouchi, R., Takeda, M., Yang, L., Wilhelmsson, U., Lundkvist, A., Pekny, M. & Chen, D. F. (2003) *Nat. Neurosci.* **6,** 863–868.
21. Baier, P. C., Schindehutte, J., Thinyane, K., Flugge, G., Fuchs, E., Mansouri, A., Paulus, W., Gruss, P. & Trenkwalder, C. (2004) *Stem Cells* **22,** 396–404.
22. Young, R. W. (1985). *Brain Res.* **353,** 229–239.