Journal of Neuroscience Research 77:334–343 (2004)

Isolation of Retinal Progenitor Cells From Post-Mortem Human Tissue and Comparison With Autologous Brain Progenitors

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The goal of the present study was threefold: to determine whether viable human retinal progenitor cells (hRPCs) could be obtained from cadaveric retinal tissue, to evaluate marker expression by these cells, and to compare hRPCs to human brain progenitor cells (hBPCs). Retinas were dissected from post-mortem premature infants, enzymatically dissociated, and grown in the presence of epidermal growth factor and basic fibroblast growth factor. The cells grew as suspended spheres or adherent monolayers, depending on culture conditions. Expanded populations were banked or harvested for analysis by RT-PCR, immunocytochemistry, and flow cytometry. hBPCs derived from forebrain specimens from the same donors were grown and used for RT-PCR. Post-mortem human retinal specimens yielded viable cultures that grew to confluence repeatedly, although not beyond 3 months. Cultured hRPCs expressed a range of markers consistent with CNS progenitor cells, including nestin, vimentin, Sox2, Ki-67, GD2 ganglioside, and CD15 (Lewis X), as well as the tetraspanins CD9 and CD81, CD95 (Fas), and MHC class I antigens. No MHC class II expression was detected. hRPCs, but not hBPCs, expressed Dach1, Pax6, Six3, Six6, and recoverin. Minority subpopulations of hRPCs and hBPCs expressed doublecortin, β-III tubulin, and glial fibrillary acidic protein, which is consistent with increased lineage restriction in subsets of cultured cells. Viable progenitor cells can be cultured from the post-mortem retina of premature infants and exhibit a gene expression profile consistent with immature neuroepithelial cells. hRPCs can be distinguished from hBPC cultures by the expression of retinal specification genes and recoverin.
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Key words: stem cells; cells, cultured; transcription factors; markers; cell differentiation

Retinal degenerative diseases, such as retinitis pigmentosa and age-related macular degeneration, now represent a major source of untreatable visual loss. These conditions are increasing in prevalence, particularly among economically developed countries with large, aging, white populations (Conley and Gorin, 2003). Currently available therapeutic measures are of limited efficacy and do not replace neurons lost during the course of the disease process. For instance, macular laser treatment can mitigate complications related to subretinal neovascularization yet cannot reverse the progressive deterioration in visual function associated with ongoing photoreceptor cell death. Novel strategies under investigation for treatment of degenerative retinal conditions include pharmacological prolongation of photoreceptor survival and transplantation of donor cells to the diseased retina. In recent years, considerable attention has been devoted to both of these strategies, although clinical success has proved elusive (Berson and Jakobiec, 1999).

The goal of retinal transplantation as a treatment is the replacement of dead or diseased host cells with healthy, integrated donor cells. Work over the last several decades in animal models has resulted in a number of advances in this area. Embryonic retinal tissue has been shown to survive transplantation to the brain of immature rats, where the graft develops a recognizable laminar organization, makes projections to host visual centers (McLoon and Lund, 1980), and is capable of relaying luminance information to the host brain (Klassen and Lund, 1987;

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Received 4 March 2004; Revised 14 April 2004; Accepted 15 April 2004

Published online 8 June 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20183

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Coffey et al., 1989). Similar grafts survive transplantation to the eye and also develop a laminar cytoarchitecture (del Cerro et al., 1992; Seiler and Aramant, 1994). Unfortunately, robust graft–host connectivity has been difficult to achieve in intraocular models using fetal tissue grafts, owing perhaps to the formation of an intervening glial scar (Gouras et al., 1994; Kinouchi et al., 2003). Recent evidence, however, indicates that this barrier may not be insurmountable. We, and others, have shown that transplanted neural progenitor cells exhibit widespread migration into the diseased host retina. This intraretinal migration is associated with the elaboration of numerous donor processes within the host neuropil, without apparent hindrance from host reactive processes (Takahashi et al., 1998; Young et al., 2000; Blixt Wojciechowski et al., 2004).

Brain progenitor cells (BPCs) can be isolated from the mammalian central nervous system (CNS) and grown in large numbers ex vivo with specific mitogens (Reynolds et al., 1992). BPCs have been referred to as a type of tissue-specific stem cell, because they exhibit the key features of self-renewal and multipotency, the latter reflected in their capacity to generate neurons, astrocytes, and oligodendrocytes. BPCs have now been derived from the brain of a number of mammalian species including, mouse, rat, dog (Milward et al., 1997), pig (Smith and Blakemore, 2000), and human (Svendsen et al., 1996). In addition, we have successfully cultured BPCs from postmortem human brain (Palmer et al., 2001; Schwartz et al., 2003). A significant limitation in the use of BPCs in models of retinal degenerative disease relates to the observation that, although they have been shown to develop morphological features highly suggestive of rod photoreceptors, there is little evidence that BPCs can express photoreceptor-specific proteins after transplantation to the mature eye (Young et al., 2000). This problem has functional implications, in that proteins such as rhodopsin and recoverin are not only phenotypic markers but also critical for effective phototransduction. One strategy for generating functional photoreceptors is to start with progenitors isolated from the neural retina. Previously, we reported the isolation and transplantation of retinal progenitor cells (RPCs) from the neural retina of green fluorescent protein (GFP)-transgenic mice (Shatos et al., 2001). The isolation and propagation of human RPCs from aborted tissue has also been reported (Kelley et al., 1995). Here we show that human RPCs (hRPCs) can be derived from post-mortem donations of immature retinal tissue and that hRPCs differ in expression of pertinent markers from autologously derived human BPCs (hBPCs).

MATERIALS AND METHODS

Donors

Informed consent for the donation of tissues was acquired prior to tissue acquisition by the authors under the auspices of the protocol for the National Human Neural Stem Cell Resource, previously approved by the Institutional Review Board of Children's Hospital of Orange County. All tissues were acquired in compliance with NIH and institutional guidelines. Tissues used in the present study were from cases SC22, SC23, SC24, SC29, and SC30. Donors consisted of two separate sets of fraternal twins, one of mixed sex (SC22, SC23), the other both male (SC29, SC30), with an estimated age of 25 weeks of gestation. All succumbed to cardiopulmonary complications within 1–48 hr of birth. A fifth donor (SC24) was born at the same age with extensive craniofacial deformities, including exencephaly, and was unable to survive ex utero.

Progenitor Cell Isolation Procedure

While being viewed through a dissecting microscope, globes were cleaned of residual conjunctiva, orbital tissue, and rectus muscles with fine scissors. Cleaned globes were rinsed in sterile Hank's balanced salt solution (HBSS) and transferred to a disposable petri dish containing cold DMEM/F12 (both solutions from Irvine Scientific, Santa Ana, CA). With a scalpel, sharp dissection was used to incise the sclera down to choroid, grasping the optic nerve with fine forceps and beginning the incision at the scleral outlet. Multiple such cuts were made in a radial pattern around the optic nerve and, with fine scissors, each was then extended in an anterior direction to the level of the pars plana. The sclera was then peeled back as a series of attached flaps, exposing the underlying uvea. The choroid and retinal pigment epithelium (RPE) could be observed to gape and separate from the underlying retina when tangential traction was applied. By using fine scissors, the retina was then incised circumferentially around the periphery, just posterior to its insertion at the ora serrata, and pulled away from the intact vitreous body with fine forceps. The optic nerve head was completely incised, resulting in a small hole in the posterior retina. Isolated retinas were transferred to a dry disposable petri dish in a tissue culture hood, minced with dual scalpels, then transferred to a sterile container for enzymatic digestion in an incubator at 37°C. Supernatants were periodically removed in the hood and replaced with fresh digestion solution. Collected supernatants were centrifuged and cells resuspended in cell-free retinal progenitor-conditioned medium. Cells were then transferred to fibronectin-coated tissue culture flasks (175 cm^2 filter cap; Greiner) containing fresh media. Brain tissue was minced and digested in a similar fashion for the harvest of hBPCs (Schwartz et al., 2003).

Cell Culture

Cells were initially incubated in growth medium containing 10% fetal bovine serum (FBS) to promote adhesion. Within 24 hr, this was exchanged for growth medium alone. Growth medium consisted of DMEM/F12 high glucose (Irvine Scientific), supplemented with BIT 9500 (BSA/insulin/transferrin; 10% by vol.; Stem Cell Technologies, Vancouver, British Columbia, Canada), recombinant human epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2; 20–40 ng/ml; Invitrogen, Carlsbad, CA), L-glutamine, and antibiotics (penicillin, streptomycin, ciprofloxacin, gentamicin, and amphotericin C). In the case of brain-derived cultures, recombinant human platelet-derived growth factor-AB (PDGF-AB; 20–40 ng/ml; Peprotech, Rocky Hill, NJ) was also included. During subsequent culturing, the medium was exchanged every 2–3 days. Any nonadherent neurospheres present in the removed supernatant were transferred to uncoated flasks and grown sep-

TABLE I. RT-PCR Primers and Conditions*

Gene	5′ Primer	3′ Primer	Annealing temp. (°C)	Size (bp)
β-Actin	CGTGCTGCTGACCGAGGCC	TTCGTGGATGCCACAGGAC	68	522
Dach1	AGGCTTTCGACCTGTTCCTGAA	GCTGTCAGACCTGTTGGTGGAA	54	336
DCX	AATCCCAACTGGTCTGTCAAC	GTTTCCCTTCATGACTCGGCA	57	405
EGF-R	CGCAAGTGTAAGAAGTGCGAA	CGTAGCATTTATGGAGAGTGAGTCT	56	93
GFAP	ACATCGAGATCGCCACCTAC	ACATCACATCCTTGTGCTCC	64	219
Hes1	CAGCCAGTGTCAACACGACAC	TCGTTCATGCACTCGCTGA	56	307
Hes5	CGCATCAACAGCAGCATAGAG	TGGAAGTGGTAAAGCAGCTTC	62	269
Nestin	GGCAGCGTTGGAACAGAGGTTGGA	CTCTAAACTGGAGTGGTCAGGGCT	65	718
Nucleostemin	CATGACCTGCCATAAGCGGT	CAATTACTCCAACCCGAATGGC	51	745
Pax6	CCAGCCAGAGCCAGCATGCAGAACA	GGTTGGTAGACACTGGTGCTGAAACT	73	950
Recoverin	TGTGTTCCGCAGCTTCGATT	TGAGGCTCAAACTGGATCAG	61	369
Six3	AGCGGACTCGGAGCCTGTTG	AGCGCATGCCGCTCGGTCCA	66	202
Six6	GGTGGGCAACTGGTTCAAAAACC	TGTCGCTGGACGTGATGGAGATG	66	212
Sox2	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG	73	131

*When possible, primers were chosen to flank at least one intron in order to amplify preferentially transcripts rather than genomic sequences.

arately. Adherent cultures were harvested enzymatically with a trypsin/EDTA solution (Custom ATV; Irvine Scientific) prior to confluence and replated, frozen, or used for analysis. After 7 days, a subset of cells was moved to chamber slides (eight-well glass; Nalge Nunc, Naperville, IL) and cultured for an additional 3 weeks under neural differentiation conditions based on a protocol detailed elsewhere (Schwartz et al., 2003) and consisting of all-trans-retinoic acid (100 nM), brain-derived neurotrophic factor (BDNF; 20 ng/ml), neurotrophin-3 (NT3; 20 ng/ml), glial-conditioned medium (glial-CM), and 1% FBS.

RT-PCR

Total RNA was extracted from hRPCs and hBPCs by using Purescript RNA Isolation Kits (Gentra, Minneapolis, MN), following the manufacturer's protocol. To ensure that samples were free of genomic DNA contamination, total RNA was treated with DNase (DNA-free; Ambion, Austin, TX). RT was carried out with M-MLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen). Negative controls received RNA but no M-MLV. PCR was performed with 3 μl of cDNA template, 0.75 μl of forward and reverse primers (0.5 μg/μl; Qiagen, Valencia, CA; Table I), and 2 U Taq DNA Polymerase (Amersham, Arlington Heights, IL) on a Techne Genius thermocycler. An initial 4-min incubation at 94°C was followed by 30 cycles consisting of 1 min at 94°C, 1 min at the corresponding annealing temperature (Table I), and 1 min at 72°C. The final extension step consisted of 7 min at 72°C. PCR products were visualized on 2% agarose gels against a 100-bp ladder.

Immunocytochemistry

Cells were prepared for analysis according to previously established protocols (Schwartz et al., 2003). Live cells were fixed for 10 min with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) buffer (Irvine Scientific). Cells were then washed in PBS buffer + 0.05% sodium azide. A blocking solution of Tris-buffered saline (TBS), 0.3% Triton X-100, and 3% donkey serum (Jackson Immunoresearch, West Grove, PA) was applied to the cells for 15 min. The cells were then rinsed twice in TBS buffer. Primary antibodies were diluted in 250 μl TBS, 0.3% Trition X-100, and 1.5% donkey serum at experimentally determined concentrations (see Table II). Primaries were applied to the samples and left overnight at 5°C on a mechanical rocker. On the following day, the cells were rinsed once with TBS. All secondaries were donkey-derived against their respected primaries (Jackson Immunoresearch) and were diluted 1:100 in 250 μl TBS, 0.3% Trition X-100, and 1.5% donkey serum. The secondaries were applied to samples and left overnight at 5°C on a rocker. On the following day, slides were rinsed three times with 1 ml TBS for 5 min each. Coverslips were mounted with Prolong Antifade Kit (Molecular Probes, Eugene, OR). Pictures were imaged on an Olympus IX70 microscope and digitally photographed with a Microfire digital camera (Optronics, Goleta, CA) and Image Pro Plus 4.5 with AFA plugin 4.5 software.

TABLE II. Primary Antibodies for Immunocytochemistry

Antigen	Species	Supplier	Product code	Dilution
CD15	Mouse	BDPharm	559045	1:100
DCX	Goat	Santa Cruz	sc-8066	1:100
FMRP	Mouse	Chemicon	MAB2160	1:200
GD_2	Mouse	Chemicon	MAB2052	1:100
GFAP	Guinea pig	Chemicon	AB1540	1:200
Ki-67	Mouse	BDPharm	556003	1:200
Nestin	Rabbit	Chemicon	AB5922	1:200
Recoverin	Rabbit	Chemicon	AB5431P	1:100
Sox2	Goat	Santa Cruz	sc-17320	1:50
Synapsin I	Rabbit	Sigma	S193	1:100
Tubulin	Mouse	Chemicon	MAB1637	1:100
Vimentin	Mouse	Sigma	V 6630	1:200

Flow Cytometry

Samples for flow cytometry were handled in the manner described previously, with the same antibodies (Klassen et al., 2001). Briefly, cells were harvested enzymatically as a single cell suspension, incubated with labeled primary or primary and labeled secondary antibodies, and analyzed on a FACS Vantage cell sorter equipped with an Enterprise 488-nm argon laser (BD

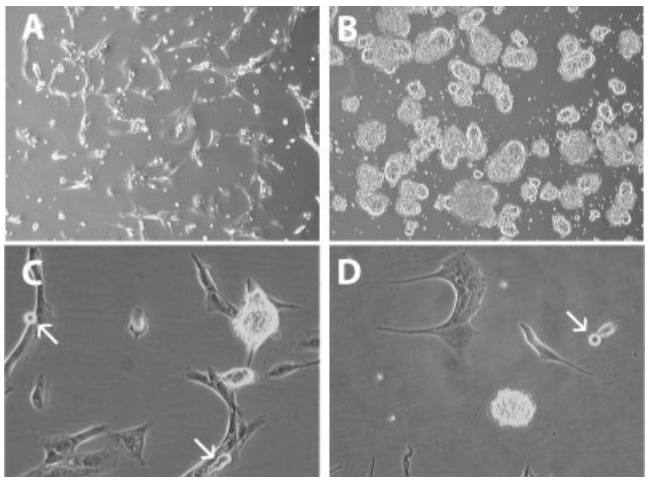

Fig. 1. Phase-contrast images of post-mortem cell cultures from immature human retina. Cells grew as a monolayer on fibronectin substrate (**A**) or as nonadherent spheres when grown in uncoated tissue culture flasks (**B**), seen here at 1 week postisolation. After 3 months in culture (**C**), the cells continued to show evidence of proliferation (arrows), although no longer growing to confluence. Viable retinal cells were also grown from post-mortem tissue obtained from a 25-week gestational age donor with a neural tube defect and major craniofacial malformations (**D**). Morphologically, these cells exhibited profiles similar to those of cells from nondeformed donors, including possible asymmetric cell division (arrow), but proliferated more slowly. A,B: ×40; C,D: ×200.

Biosciences, San Jose, CA). Color compensation was preliminarily set by using calibrite beads (BD Biosciences), and individual samples were optimized with single positive antibody labeling for NCAM (CD56), compared with IgG control (MOPC). Two-color live-gating acquisition was used to optimize settings and acquire data. Up to 30,000 events were collected and stored electronically for subsequent analysis.

RESULTS

Cell Culture

Grown under proliferation conditions, post-mortem human retinal cultures yielded viable cells that formed an adherent monolayer on fibronectin or grew in suspended clusters analogous to brain-derived neurospheres (Fig. 1A,B). The morphology within the adherent population was variable and included small, rounded profiles; medium-sized bipolar and spindle-shaped profiles; and larger cells with polygonal and multipolar morphologies. With time in culture, clusters of cells frequently extended vertically into the medium, eventually budding off as neurospheres. After 3 months, the cultures continued to exhibit evidence of ongoing cell division, including a subset of cells with Ki-67 immunoreactivity (data not shown), but no longer grew to confluence (Fig. 1C). Retinas from the donor with exencephaly and severe craniofacial malformations (case SC24) appeared thin compared with those from donors of the same developmental age. The cells derived from this tissue exhibited a microscopic appearance similar to that of the other retinal cultures but passaged more slowly (Fig. 1D).

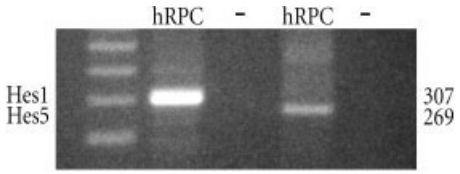

Fig. 2. Cultured human retinal progenitor cells express downstream genes of the Notch pathway. RT-PCR bands are seen corresponding to the expected product sizes for the transcription factors Hes1 and Hes5. The far left lane contains a 100-bp ladder, with the lanes to the right of the ladder showing, in sequence, Hes1 expression by hRPCs, negative control (Hes1 primers but not reverse transcriptase), Hes5 expression by hRPCs, and corresponding negative control for Hes5.

Retinal Progenitors Compared With Brain Progenitors by RT-PCR

Retina-derived populations (Fig. 2) expressed the nuclear transcription factors Hes1 and Hes5, both associated with the neurodevelopmentally regulated Notch pathway. Comparison of retina- and brain-derived cultures (Fig. 3) revealed that both populations expressed the neurodevelopmental markers doublecortin (DCX), epidermal growth factor receptor (EGF-R), nestin, and nucleostemin, whereas Pax6 was seen only in RNA extracted from retinal populations. Additional genes expressed by both retinal and brain cultures included Sox2 and glial fibrillary acidic protein (GFAP), whereas other genes expressed by retinal cultures alone included the eye specification genes Dach1, Six3, and Six6, as well as the retinal marker recoverin (Fig. 3). All RT-PCR results pertain to case SC30, whereas the positive results for DCX, EGF-R, GFAP, nestin, nucleostemin, and recoverin also apply to pooled RNA from cases SC22 and SC23.

Phenotypic Markers by Fluorescence Immunocytochemistry

Immunocytochemical analysis of hRPCs grown under proliferation conditions (Fig. 4A–E,G–I) and differentiation conditions (Fig. 4F,J–L) demonstrated expression of a range of developmental and mature markers. Neurodevelopmental markers that showed a high degree of colocalization included the intermediate filament nestin, the transcription factor Sox2, and the surface marker GD_2 ganglioside (Fig. 4A). A subset of cells expressed the neurodevelopmental marker CD15 (Lewis X; Fig. 4B). Distinct subpopulations of cultured cells expressed the neuronal lineage marker β-III tubulin and the glial lineage marker GFAP (Fig. 4C). Also expressed by distinct subpopulations were the neuroblast marker DCX and the photoreceptor marker recoverin (Fig. 4D). The proliferation marker Ki-67 colocalized with nestin and Sox2, indicating a high level of mitotic activity in RPCs grown under proliferation conditions (Fig. 4E). Analysis of intermediate filament expression showed that, in addition to nestin, vimentin and GFAP were expressed and that nestin colocalized with vimentin more frequently than with GFAP (Fig. 4G–I).

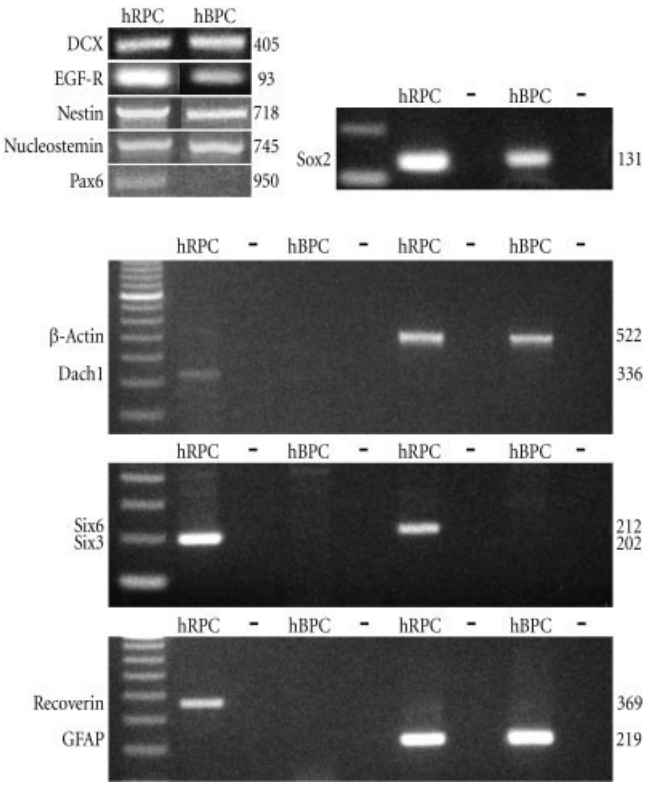

Fig. 3. Comparison of human progenitor cells from retina and brain with RT-PCR. Markers expressed by both retina- and brain-derived populations include DCX, EGF-R, nestin, and nucleostemin. In contrast, Pax6 was seen only in RNA extracted from hRPCs. Sox2 was also expressed by both retina and brain progenitors, as was β-actin as a positive control, whereas Dach1 was differentially expressed by hRPCs compared with hBPCs. Other transcripts differentially expressed by retinal cultures included the developmental genes Six3 and Six6 and the mature retinal marker recoverin, whereas GFAP was expressed by both. Products are compared with a 100-bp ladder with lanes arranged, from the left, in the following sequence for each gene: retina, retina negative control, brain, brain negative control.

When hRPCs were cultured under proneuronal differentiation conditions (retinoic acid, BDNF, NT3, glial-CM, FBS), several changes in marker expression were evident. Treated cultures lost Ki-67 immunoreactivity, Sox2 was relocated to a perinuclear distribution, nestin was down-regulated, and cells expressed fragile X mental retardation protein (FMRP; Fig. 4F). Treated cultures also exhibited widespread expression of the neuronal lineage markers DCX, β-III tubulin, and synapsin, which generally colocalized to the same cells (Fig. 4J–L). In terms of subcellular localization, synapsin and DCX were evident at the tips of neuritic processes, whereas β-III tubulin appeared to be excluded from this subcellular domain (Fig. 4J–L,M–O). All imunocytochemical results pertain to case SC30, except the data on Sox translocation (Fig. 4F), which are from cases SC22/SC23. Positive immunoreactivity for nestin, GD_2 ganglioside, Ki-67, Sox2, GFAP, and vimentin was common to all cases examined (SC22, SC23, SC30).

Surface Markers by Flow Cytometry

Flow cytometry was used to evaluate the expression of surface markers by hRPCs. By using this method (Fig. 5), it was shown that RPCs express variable amounts of GD_2 ganglioside, which is consistent with the immunocytochemical findings for this marker. hRPCs also expressed the tetraspanins CD9 (MRP-1) and CD81 (TAPA-1), the cell adhesion molecules CD54 (ICAM) and CD56 (NCAM), and the apoptosis-related receptor CD95 (Fas). In terms of transplantation antigens, hRPCs expressed MHC class I antigens, including both heavy chain (HLA-ABC) and β2-microglobulin, but did not express detectible MHC class II (HLA-DR, DP, DQ) antigens. Flow cytometric analysis of hRPCs from the donor with multiple cranial abnormalities showed that these cells also expressed GD_2 ganglioside and MHC class I (Fig. 5, Table III). All flow cytometric data pertain to cases SC22, SC23, and SC24 (to the extent shown in Fig. 5).

DISCUSSION

Human progenitor cells can be derived from retinal tissue obtained post-mortem from premature infants. These cells were maintained in culture for 3 months and expressed a host of immature markers consistent with undifferentiated neuroepithelial cells of the developing retina. In addition, subpopulations of cells expressed neural, photoreceptor, and glial markers consistent with phenotypic multipotency. Post-mortem hRPCs expressed many markers in common with autologous brain progenitor cells yet can be distinguished from the latter based on differential expression of retinal specification genes and recoverin.

The derivation of viable progenitor cells from the post-mortem human retina extends our previous work showing the derivation of similar cells from the post-mortem human brain (Palmer et al., 2001; Schwartz et al., 2003). Although considerable effort was made to minimize the time between death and cell isolation, viable cultures were obtained from human brain and retina following post-mortem intervals in excess of 24 hr. A major determinant of the proliferative capacity of hRPC cultures is donor age, with younger donors being preferable, particularly premature infants. Although earlier donations can be obtained in the setting of intrauterine fetal demise, these cases are less likely to yield good results, because the effective post-mortem interval is frequently long and the tissue poorly preserved. Retinal progenitors have been previously obtained from abortuses; however, the use of post-mortem tissue makes possible the study of hRPCs obtained at more mature developmental time points. In addition, a major advantage of using post-mortem tissue, as opposed to aborted tissue, is that the former method of donation is free of ethical controversy.

The identification of the cells obtained as retinal progenitors is based on tissue of origin, method of isolation, and marker expression. The cells were derived from the neural retina of second-trimester donors, a time when clearly recognizable retinal layers have formed but cellular differentiation is incomplete (Mann, 1950). The method

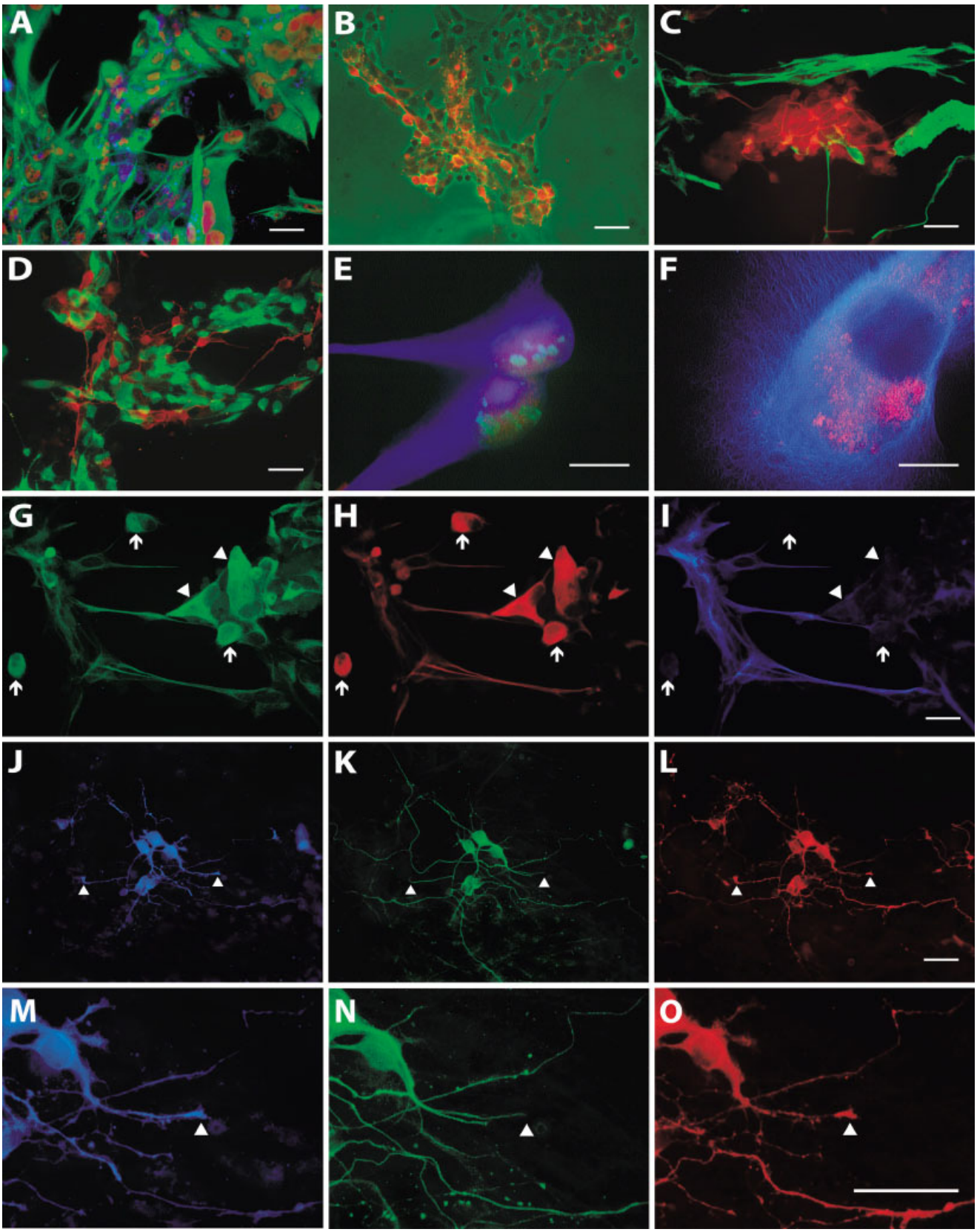

Figure 4.

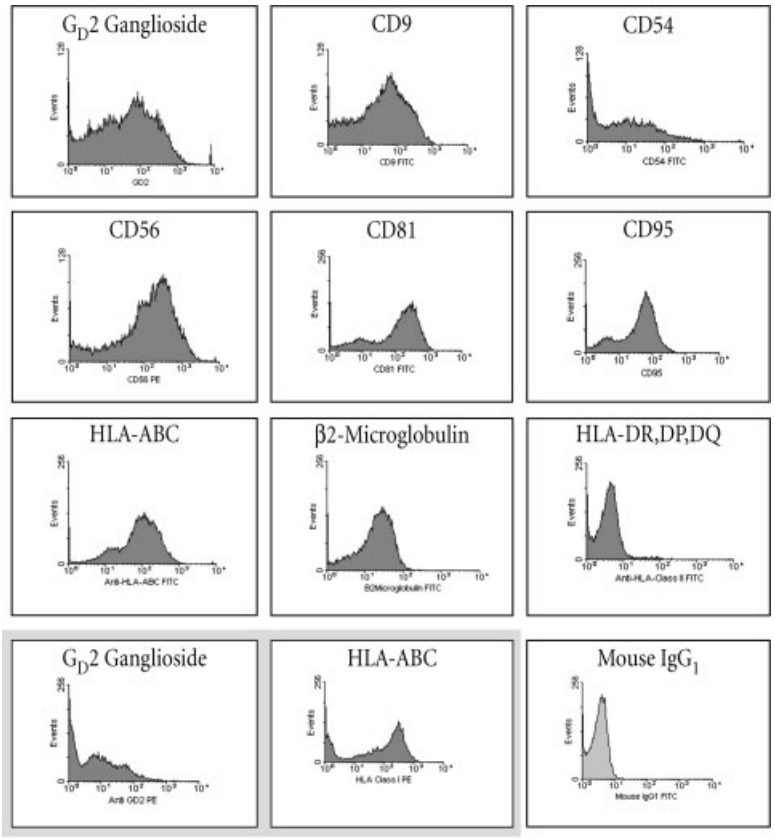

Fig. 5. Flow cytometric evaluation of surface markers on human retinal progenitor cells. Cells expressed GD_2 ganglioside, CD9 (MRP-1, a tetraspanin), CD54 (ICAM), CD56 (NCAM), CD81 (TAPA-1, a tetraspanin), and CD95 (Fas). hRPCs also expressed MHC class I transplantation antigens, including both heavy chain (HLA-ABC) and β2-microglobulin epitopes, but did not express detectable MHC class II (HLA-DR, DP, DQ). Data from the RPCs derived from donor with mutiple congenital craniofacial abnormalities (case SC24) are shown in the first two frames of the bottom row (enclosed by gray border). These cells expressed GD_2 ganglioside and MHC class I (HLA-ABC). Data are compared with mouse MOPC IgG (final frame), used as a negative control.

used to isolate these cells was adapted from neuroselective methods developed for culturing CNS stem cells from the brain (Reynolds et al., 1992), spinal cord (Shihabuddin et al., 1997), and retina (Shatos et al., 2001) of rodents as well as the brain of humans (Palmer et al., 2001; Schwartz et al., 2003). Passaged cells expressed a number of immature markers, including putative neural stem cell markers and retinal specification gene products. Although the currently recognized method for establishling multipotency is clonal derivation, followed by differentiation, these cells proliferated poorly when seeded at low density, so clonal derivation was not successful. Nevertheless, analysis of marker expression provided evidence that these cultures give rise to cells of neuronal lineage (DCX, β-III tubulin, synapsin), including photoreceptor lineage (recoverin) and glial lineage (GFAP). Furthermore, extensive retinal lineage studies in other models previously established that multipotency is maintained up to the final cell division (Turner

Fig. 4. Immunolocalization of phenotypic markers in retinal progenitor cell cultures. **A–E,G–I:** Proliferation conditions. **F,J–L:** Differentiation conditions. A: Nestin (green) staining showed a cytoplasmic pattern, Sox2 (red) a nuclear pattern, and GD_2 ganglioside (blue) a surface pattern consisting of discrete, punctate foci of variable size and number. B: CD15 immunoreactivity (red) was variable and most evident on a subset of cultured cells with small, rounded profiles (shown against phase contrast). C: Distinct subpopulations within hRPC cultures expressed either the neuronal marker β-III tubulin (red) or the glial marker GFAP (green). D: Other subpopulations could be distinguished by expression of the neuroblast marker DCX (red) compared with the photoreceptor marker recoverin (green). E: Nestin (blue) and Sox2 (red) colocalized with Ki-67 (green) in hRPCs grown under proliferation conditions. F: Under proneuronal differentiation conditions, there was an absence of Ki-67 staining, whereas Sox2 (red) was still detectable but now assumed a perinuclear distribution together with cytoplasmic expression of FMRP (blue). G–I: Series analysis of intermediate filaments under proliferation conditions showed that nestin (G), vimentin (H), and GFAP (I) were all expressed in hRPC cultures. Among these, nestin (green) and vimentin (red) showed a high degree of colocalization (arrows, arrowheads), whereas GFAP (blue) was associated with a subset of flat, extended profiles and generally excluded from smaller, rounded profiles (arrows) that expressed nestin and vimentin (note same field of view for G–I). J–L: Series analysis of neuronal markers in hRPCs cultured under differentiation conditions showed expression of DCX (J), β-III tubulin (K), and synapsin (L), with a high degree of cellular colocalization for all three markers (note same field of view for J–L). At the subcellular level, synapsin (red) and DCX (blue) were frequently present at the tips of β-III tubulin$^+$ processes, although tubulin itself (green) was not (arrowheads point to the same tips in each frame of J–L). **M–O:** This finding is more evident in a digitally enlarged portion of the preceding series showing one of the indicated neuritic tips in which the relative staining for DCX (blue, M), β-III tubulin (green, N), and synapsin (red, O) can be compared (note same field of view for M–O). A–E,G–O: ×40; F: ×100. Scale bars = 50 μm in A–D, 10 μm in E,F; bar in I = 50 μm for G–I; bar in L = 50 μm for J–L; bar in O = 50 μm for M–O.

TABLE III. Summary of Markers Expressed in hRPC Cultures, Along With Molecular Category and Method of Identification

Marker	Molecular category	Method of identification
CD9	Tetraspanin	FACS
CD15	Carbohydrate moiety	ICC
CD81	Tetraspanin	FACS
Dach1	Transcription factor	PCR
Doublecortin	Microtubule-associated	PCR, ICC
EGF-R	Growth factor receptor	PCR
Fas	Death (apoptosis) receptor	FACS
FMRP	mRNA binding protein	ICC
GD_2	Ganglioside	ICC, FACS
GFAP	Intermediate filament	PCR, ICC
Hes1	Transcription factor	PCR
Hes5	Transcription factor	PCR
ICAM	Adhesion molecule	FACS
Ki-67	Cell cycle-related	ICC
MHC I	Transplantation antigen	FACS
NCAM	Adhesion molecule	FACS
Nestin	Intermediate filament	PCR, ICC
Nucleostemin	Cell cycle-related	PCR
Pax6	Transcription factor	PCR
Recoverin	Phototransduction	PCR, ICC
Six3	Transcription factor	PCR
Six6	Transcription factor	PCR
Sox2	Transcription factor	PCR, ICC
β-III Tubulin	Cytoskeletal protein	ICC
Vimentin	Intermediate filament	ICC

and Cepko, 1987). Taken in combination with these findings, our data are most consistent with the interpretation that the immature (nestin, Sox2), highly proliferative (Ki-67) neuroepithelial cells in the present study were multipotent retinal progenitors. In addition, the data suggest that, although neural progenitors can be derived from different regions of the CNS, the progenitors so derived are likely to differ in phenotypic potential, an important consideration for developmental studies as well as in transplantation paradigms.

The literature contains fewer reports specifically devoted to the derivation of progenitor cell cultures from the mammalian retina than from the brain. Considerable attention has, however, been directed toward understanding the role of progenitor cells in retinal development. Much of this work has been carried out on intact embryos or by using dissociated embryonic retinal cultures. Such studies include analysis of the influence of growth factors on rat retinal progenitors (Guillemot and Cepko, 1992), investigations of the timing of fate determination in embryonic chick retinal cells (Belecky-Adams et al., 1996), and studies of the potential for cell fate respecification (James et al., 2003). In addition, we have previously reported the isolation of retinal progenitor cells from the neural retina of the early postnatal mouse (Shatos et al., 2001), and another group has reported the isolation of retinal stem cells from the pigmented epithelium of the mouse ciliary body (Tropepe et al., 2000). In terms of human cells, there is a prior report of the amplification of retinal progenitors in culture (Kelley et al., 1995), although notable differences exist between that study and the current one. First, the cells in that study were obtained from aborted embryonic and fetal tissue obtained considerably earlier in development (6–13 weeks of gestation). The 12–19-week difference in developmental age at the time of harvest may account in some part for the 7-weeks-longer growth of those cells in culture (up to 300 days) than we report here (up to 90 days). In addition, the donated tissue in that study was collected without a prolonged post-mortem interval. Nevertheless, cells obtained by both methods were propagated in culture and shown to be capable of expressing photoreceptor and other neuroretinal markers.

The human retinal progenitors of the present study expressed a number of markers consistent with immature neuroepithelial cells, including nestin, vimentin, Ki-67, nucleostemin, EGF-R, Sox2, GD_2 ganglioside, and, to some extent, CD15 and DCX (Figs. 3–5). In addition, hRPCs expressed genes associated with retinal development, including Hes1, Hes5 (Fig. 2), Dach1, Pax6, Six3, and Six6 (Fig. 3). Individually, none of these genes is specific for RPCs, in that each is expressed elsewhere than in the retina at some point in development. For instance, Pax6 and Six3 are expressed in the brain, albeit in a developmentally restricted manner (Andrews and Mastick, 2003; Lagutin et al., 2003), and Dach1 and Six6 are expressed during pituitary development (Li et al., 2002). Taken together, however, the gene expression profile reported here is highly suggestive of cells originating from the developing retina, a point underscored by comparison with cells from the brain of the same donor. Specifically, autologous hBPCs also expressed nestin, vimentin, Ki-67, nucleostemin, EGF-R, Sox2, GD_2 ganglioside, and, to a variable extent, CD15 and DCX (Fig. 3; Schwartz et al., 2003) but did not express Dach1, Pax6, Six3, or Six6 (Fig. 3). Examination of a broad profile may be particularly useful when encountering unexpected variability for a given gene, as has been reported for expression of Pax6 by neural stem cells in culture (Santa-Olalla et al., 2003). Furthermore, given the importance of transcription factors to the regulation of cell fate, it is likely that comprehensive evaluation of neural transcription factor expression will be of interest when characterizing ontogenetic status and phenotypic potential.

Nuclear transcription factors (NTFs) identified in the current study as expressed by hRPCs include Dach1, Hes1, Hes5, Pax6, Six3, Six6, and Sox2. For these, previous studies have shown that Sox2 expression is associated with neural progenitors throughout the developing vertebrate CNS, and it has been considered a marker of neural stem cells (Zappone et al., 2000; Cai et al., 2002; D'Amour and Gage, 2003; Graham et al., 2003; Schwartz et al., 2003), although it is also expressed in embryonic stem cells and non-CNS tissues, such as the developing lens (Kamachi et al., 2001). Sox genes play a role in neural development that appears to be highly conserved, in that the putative *Drosophila* ortholog SoxNeuro also shows widespread expression in the nacent neuroectoderm of the

embryonic fruit fly (Buescher et al., 2002). Consistently with a pervasive neurodevelopmental role, Sox2 expression was widespread in both brain- and retina-derived progenitor cultures. Hes1 and Hes5 are hairy/Enhancer of split-related genes that are transcriptional repressors of the basic helix-loop-helix (bHLH) family and downstream effectors of Notch signaling. These factors forestall expression of proneural NTFs, such as Mash1 and Ngn1, thereby maintaining phenotypic immaturity during early neural development (Cau et al., 2000). Dach1, Pax6, Six3, and Six6 are homologs of the *Drosophila* eye specification genes dachshund, eyeless, and sine oculis, along with the related gene optix, and all participate in vertebrate retinal development (Kumar and Moses, 2001). To date, mutations in Pax6 and Six6 have been associated with human eye disease (Jordan et al., 1992; Gallardo et al., 1999), Six3 is known to be expressed in the human eye (Leppert et al., 1999), and Dach1 has been implicated in nonocular human disease (Ayres et al., 2001; Ozaki et al., 2002). Here we report the expression of all these genes by cultured hRPCs, supporting their relevance in the context of human retinal development.

Cytoskeletal-associated proteins represent another class of marker known to be useful for the characterization of neural cultures. DCX is a microtubule-binding protein transiently expressed during CNS development and known to be expressed by migrating neuroblasts (Gleeson et al., 1999; Bai et al., 2003; Brown et al., 2003). DCX appears to be active in the leading processes of growing neurites, and mutations of this gene are associated with abnormalities of cortical development, such as lissencephaly (Friocourt et al., 2003). Previously, we have shown that DCX is expressed by a nestin$^-$, GFAP$^-$, Ki-67$^-$ minority within forebrain progenitor cultures under proliferation conditions (Schwartz et al., 2003), and we now extend these results to cultured progenitors from the human retina (Figs. 3, 4). Moreover, in both types of cultures, DCX$^+$ cells have small, rounded somata and two or three long, thin, sparsely branched processes (Fig. 4D). The characteristic morphology of DCX$^+$ cells stands in contrast to the broader range of cellular morphologies present in CNS progenitor cultures, for example, the majority of nestin$^+$, vimentin$^+$, and GFAP$^+$ profiles (Fig. 4A,C,G–I). Because these cells are likely to be lineage-restricted protoneuronal precursors, rather than multipotent progenitor cells, our data suggest that phenotypic heterogeneity is a feature of mammalian CNS progenitor cultures. The protoneuronal status of DCX$^+$ cells is supported here by coexpression of β-III tubulin and synapsin under differentiation conditions (Fig. 4J–L).

In the present study, we have also shown that hRPC cultures contain an additional subpopulation of small cells, in this case expressing the photoreceptor marker recoverin. These recoverin$^+$ cells were more numerous than the DCX$^+$ cells and the two subpopulations were seen to aggregate in culture without coexpression of these markers (Fig. 4D). The presence under proliferation conditions of subpopulations expressing DCX, recoverin, β-III tubulin, and GFAP (Fig. 4C,D) among the majority of cells expressing the immature markers nestin, Sox2, and vimentin suggests a tendency toward continuous differentiation in these cultures. Indeed, DCX- and GFAP-expressing subpopulations were also present in the brain-derived human progenitors, as seen here (Fig. 3) and as previously reported (Schwartz et al., 2003). In another study, opsin expression was reported following short-term treatment of human fetal brain progenitors with transforming growth factor-β3 (TGF-β3; Dong et al., 2003). Whether such cells are photosensitive and whether similar treatment is capable of inducing opsin expression in the retinal progenitors of the present study remains to be determined. Further studies will also be necessary to determine whether these findings are specific to cells harvested at this developmental time point or can be generalized to a wider range of CNS progenitors, either from humans or from other species. Also of interest is the potential of hRPCs to integrate and differentiate into retinal neurons following transplantation, particularly in animal models of photoreceptor loss.

ACKNOWLEDGMENTS

The authors thank Drs. Marie Shatos and Tasneem Zahir for technical assistance. This study was supported by the American Cell Therapy Research Foundation/Stem Cell Research Foundation (H.K.), the CHOC Foundation, Guilds, and Padrinos (H.K., P.H.S.), the United Mitochondrial Research Foundation (P.H.S.), the Siegal Foundation (M.J.Y.), the Minda de Gunzburg Center for Retinal Transplantation (M.J.Y.), the NINDS (NS044060-01S1; H.K.), and the NEI (EY09595; M.J.Y.).

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