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Retinal Incorporation and Differentiation of Neural Precursors Derived from Human Embryonic Stem Cells

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Key Words. Human embryonic stem cells • Differentiation • Retina • Transplantation • Photoreceptors

ABSTRACT

Retinal and macular degenerations are a major cause of blindness. Cell transplantation is a possible therapeutic approach for the replacement of degenerating retinal cells. Here, we studied the potential of human embryonic stem cells (hESCs) to survive, integrate, and differentiate into retinal cells after intraocular transplantation. Highly enriched cultures of neural precursors (NPs) expressing transcripts of key regulatory genes of retinal development were developed from the hESCs. After spontaneous differentiation in vitro, the NPs gave rise to progeny expressing markers of retinal progenitors and photoreceptor development, though this was uncommon and cells expressing markers of mature photoreceptors were not observed. After transplantation into rat eyes, the NPs survived for 16 weeks, migrated large distances, and integrated in the host retina. Teratoma tumors were not observed. Human cells expressing rhodopsin, blue cone opsin, and neural retina leucine zipper transcription factor were observed in subretinal grafts, but not within vitreal and inner retinal grafts. The results suggest that hESCs have the potential to differentiate into retinal cells and that the subretinal microenvironment supports their differentiation toward a photoreceptor fate. This may be the first step toward further developments that eventually may allow the use of hESCs for transplantation in retinal degenerations. STEM CELLS *2006;24:246–257*

INTRODUCTION

Retinal and macular degenerations are major causes of blindness in all age groups. Retinitis pigmentosa (RP), a group of genetic diseases leading to progressive photoreceptor degeneration, affects approximately 1.5 million patients worldwide and is the most common cause of blindness in people under 70 years of age in the Western world [1]. Among the older age group, age-related macular degeneration (AMD) is the most common cause of blindness [2]. Various treatment modalities to prevent, delay, or repair retinal cell degeneration are being investigated, including nutritional supplementation, gene therapy, the use of trophic factors, and even the development of means of artificial vision [1, 3, 4]. However, the current clinical reality is that in many patients, retinal function is ultimately lost.

Cell therapy is considered a potential therapeutic approach that may restore or sustain retinal function and prevent blindness in patients with retinal degeneration [5]. As such, retinal engraftment of embryonic or fetal retinal tissue, brain-derived

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1066-5099/2006/$20.00/0 doi: 10.1634/stemcells.2005-0009

neural precursors (NPs), nonhuman primate and rodent embryonic stem cells (ESCs), bone marrow–derived stem cells, and retinal progenitors have been attempted [1, 6–16]. It has been shown in a number of animal models that survival, differentiation, and even limited connectivity of such grafts with the host retina can be achieved, thus supporting the concept of cell therapy for the injured or degenerating retina. Effective clinical application of this approach is as yet unrealized, mainly because of the difficulty in achieving functional integration of grafted tissue.

Whereas the use of human fetal tissue is limited by ethical considerations and insufficient supply, human ESCs (hESCs) may serve as an unlimited source of neural cells for retinal transplantation therapy. We have developed methods that allow the derivation of highly enriched (>95%), expandable populations of developmentally competent NPs from hESCs [17]. In this study, we examined their integration, differentiation after intraocular transplantation, as well as their potential to adopt a retinal fate. We show that the NPs express key regulatory genes of ocular and retinal differentiation in vitro. Furthermore, after transplantation into the subretinal space, the NPs differentiate into cells expressing retina- and photoreceptor-specific markers. To the best of our knowledge, this is the first report showing the potential of hESCs to differentiate toward a photoreceptor fate.

MATERIALS AND METHODS

Derivation and Culture of NPs

hESCs (HES-1 cell line) with a stable, normal (46XX) karyotype were cultured either in the presence of serum on a mouse embryonic fibroblast feeder layer or in the absence of serum on human foreskin feeders, as previously described [17, 18]. The cells were genetically modified by a lentiviral vector expressing enhanced green fluorescent protein (eGFP) under the human elongation factor 1 α (EF1α) promoter, according to our described protocol [19]. The percentage of infected cells and intensity of transgene expression were analyzed on a FACS Calibur system (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). To induce neural differentiation, clumps of undifferentiated hESCs were plated on fresh mitotically inactivated mouse feeders and cultured for 8 days in medium comprised of Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com) containing glucose 4.5 g/l without sodium pyruvate, supplemented with 10% fetal bovine serum (HyClone, Logan, UT, http://www.hyclone.com), 0.1 mM beta-mercaptoethanol, 1% nonessential amino acids, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco-BRL), and 500 ng/ml noggin (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). The cells were then further cultured in the same medium in the absence of noggin for an additional 5 days. At this time, 70%–90% of the colonies differentiated almost uniformly into tightly packed small cells with a uniform gray opaque appearance under dark-field stereomicroscopy. Patches containing approximately 150 cells each were dissected from the gray opaque areas using a razor blade (surgical blade no. 15) and replated in serum-free medium that consisted of DMEM/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 U/ml, and streptomycin 50 μg/ml (Gibco-BRL) and that was supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems Inc.). The clusters of cells developed into round spheres that were subcultured once a week as previously described [17]. The medium was replaced twice a week.

Immunofluorescent Studies In Vitro

Standard protocols were used for the immunophenotyping of disaggregated progenitor cells and differentiated cells after fixation with 4% paraformaldehyde. Primary antibodies were detected by using goat anti-mouse immunoglobulin M (IgM) conjugated to Texas Red (1:100), goat anti-rabbit Ig conjugated to Texas Red (1:500), goat anti-mouse IgG conjugated to CyTH3 (1:500), rhodamine Red^{TM}-conjugated donkey anti-goat IgG (1:500; all from Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, http://www.jacksonimmuno.com), and swine anti-rabbit Ig conjugated to fluorescein isothiocyanate (FITC) (1:50; DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.dk). Proper controls for primary and secondary antibodies were used to rule out nonspecific staining or cross-reactivity between antibodies.

To characterize the immunophenotype of cells within the aggregates, spheres cultivated for 4 weeks were partially disaggregated by mechanical means, and the resulting small clumps and single cells were plated in serum-free medium, as described above, on poly-D-lysine (30–70 kDa, 10 μg/ml) and laminin (4 μg/ml; both from Sigma, St. Louis, http://www.sigmaaldrich.com). The cells were fixed after 18 hours and examined for the expression of NCAM (mouse IgG 1:10; DakoCytomation), nestin (rabbit antiserum, 1:25, a kind gift of Dr. Ron McKay, or rabbit anti-human 1:100–200; Chemicon International, Inc., Temecula, CA, http://www.chemicon.com), A2B5 (mouse clone 105 1:20; American Type Culture Collection, Manassas, VA, http://www.atcc.org), and polysialic acid (PSA)–NCAM (mouse undiluted; Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww, or mouse IgM 1:200; Chemicon International, Inc.).

To induce differentiation, spheres that were 4 weeks in culture were disaggregated into small clumps and plated on poly-D-lysine and laminin in serum-free growth medium (as described above) without supplementation of growth factors for 2–3 weeks. The medium was supplemented with the survival factors neurotrophin-3 (NT3) 10 ng/ml, NT4 20 ng/ml, and brain-derived neurotrophic factor (BDNF) 10 ng/ml (all human recombinants from R&D Systems Inc.) when the expression of retinal progenitor markers was examined. Differentiated cells were analyzed for the expression of β-tubulin III (mouse IgG 1:2,000; Sigma), neurofilament 70 (mouse IgG_1, 1:50; DakoCytomation), neurofilament 160 (mouse IgG_1, 1: 50; Chemicon International, Inc.), microtubule-associated protein 2ab (MAP2ab) (rabbit polyclonal, 1:100; Chemicon International, Inc.), glial fibrillary acidic protein (GFAP) (rabbit polyclonal, 1:200; DakoCytomation), γ-aminobutyric acid (GABA) (rabbit polyclonal, 1:500; Sigma), glutamate (rabbit polyclonal, 1:2,000; Sigma), Pax6 (mouse monoclonal IgG_1, 1:100; Developmental Studies Hybridoma Bank), Chx10 (rabbit polyclonal, 1:500, kindly provided by Roderick R. McInnes, Toronto), cone-rod homeobox (CRX) (rabbit polyclonal, 1:2,500, kindly provided by Cheryl Y. Gregory-Evans, London), rhodopsin (mouse IgG_1 1:500; Lab Vision Corporation, Fremont, CA,

http://www.labvision.com), blue-sensitive opsin (goat polyclonal, 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com), neural retina leucine zipper (NRL) (rabbit polyclonal, 1:2,000; generously donated by Dr. Anand Swaroop, University of Michigan, Ann Arbor, MI), serotonin (rabbit polyclonal, 1:1,000; Sigma), and tyrosine hydroxylase (TH) (mouse $IgG_{1,}$ 1:500; Sigma). For quantitative analysis of marker expression, 200–500 cells were scored within random fields (at ×400) for the expression of the markers, and the experiments were repeated at least three times.

Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from (a) hESCs grown under serum-free conditions (1 week after passage), (b) free-floating spheres after 4 weeks in culture, and (c) differentiated cells growing from the spheres at 2 weeks after plating in differentiation-inducing conditions, as detailed above. The medium used to induce differentiation was supplemented with a combination of ascorbic acid 400 μM (Sigma) and the survival factors NT3 10 ng/ml, NT4 20 ng/ml, and BDNF 10 ng/ml (all human recombinants from R&D Systems Inc.). Total RNA was isolated using RNA STAT-60 solution (Tel-Test, Friendswood, TX, http://www.isotexdiagnostics.com) or TRI-reagent (Sigma) followed by treatment with RNase-free DNase (Ambion, Inc., Austin, TX, http://www.ambion.com). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcription (RT) and oligo (dT) as a primer, according to the manufacturer's instructions (Promega Corporation, Madison, WI, http://www.promega.com). To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Levels of different mRNAs expressed by neural spheres and differentiated cells were compared with that in undifferentiated hESCs. Polymerase chain reaction (PCR) was carried out using standard protocols with Taq DNA Polymerase (Gibco-BRL). Amplification conditions were as follows: denaturation at 94°C for 15 seconds, annealing at 55°C–64°C for 30 seconds, and extension at 72°C for 45 seconds. The number of cycles varied between 18 and 40, depending on particular mRNA abundance. Primer sequences (forward and reverse 5′–3′) and the length of the amplified products were as follows:

Oct4 (CGTTCTCTTTGGAAAGGTGTTC, ACACTCGGACCACGTCTTTC; 320 bp)

Six3 (CAAGTCCACACACACTCCCAC, CGTCATGCAGGTGGGGTGC; 254 bp)

Six6 (CCTGCAGGATCCATACCCTA, TGATGGAGATGGCTGAAGTG; 272 bp)

Rx (CTGAAAGCCAAGGAGCACATC, CTCCTGGGAATGGCCAAGTTT; 408 bp)

Pax6 (AACAGACACAGCCCTCACAAACA, CGGGAACTTGAACTGGAACTGAC; 274 bp)

Lhx2 (CCAAGGACTTGAAGCAGCTC, TGCCAGGCACAGAAGTTAAG; 285 bp)

Chx10 (GGCGACACAGGACAATCTTT, ATCCTTGGCTGACTTGAGGA; 281 bp)

Crx (GTGTGGATCTGATGCACCAG, TGAGATGCCCAGAGGGTCT; 352 bp)

Nrl (AGAGCGCCTTCTGGTCCTAG, GCATCTCGGATAGAGGTCCT; 421 bp)

Recoverin (TGTGTTCCGCAGCTTCGATT, TGAGGCTCAAACTGGATCAG; 368 bp)

OpsinB (GGTCACTGGCCTTCCTGG, TGCAGGCCCTCAGGGATG; 176 bp)

GAPDH (AGCCACATCGCTCAGACACC, GTACTCAGCGCCAGCATCG; 301 bp).

Subretinal Transplantation of NPs

Animals. A total of 23 adult (body weight 230–250 g) and 38 newborn (2–3 days old) outbred Sabra rats were used in the present study, in three separate experimental cycles. All animal experiments were conducted according to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the institutional committee for animal research. Newborn rats were kept with their mothers until 3–4 weeks of age.

For intraocular injections, adult animals were anesthetized with Ketamine HCl (100 mg/kg Ketalar; Parke Davis, U.K., http://www.parke-davis.com), injected intraperitoneally in combination with the relaxing agent Xylazine (2.0 mg/kg). Local anesthetic drops (Benoxinate HCl 0.4%; Fischer Pharmaceuticals, Tel Aviv, Israel, http://www.dr-fischer.com) were administered. In newborn rats, injections were executed under ether anesthesia (ether absolute; Bio-Lab Ltd., Jerusalem, Israel, http://www.bio-lab.co.il) in combination with local anesthetic drops. The pupils were dilated with Tropicamide 0.5% (Mydramide; Fisher Pharmaceuticals, Tel Aviv, Israel) and Phenylephrine HCl 2.5% (Fisher Pharmaceuticals, Israel). Animals were kept warm during and after the procedure, using a heating lamp. After transplantation, all animals received the immunosuppressive agent cyclosporine A (50 mg/ml Sandimmune; Novartis Pharma AG, Basel, Switzerland, http://www.novartis.com) in their drinking water at a concentration of 210 mg/l.

Intraocular Injections. Glass capillaries were pulled from filaments (1.2 × 0.68 mm; A-M Systems, Inc., Everett, WA, http://www.a-msystems.com) on a P97 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA, http://www.sutter.com). After systemic and local anesthesia, eyes were exposed; in newborns, gentle dissection was performed to separate the closed eyelids. Under visualization of a dissecting microscope (Stemi SV 11; Carl Zeiss, Jena, Germany, http://www.zeiss.com), the glass capillary, coupled to a pneumatic Pico-injector (PLI-100; Medical System Corp., Greenvale, NY, http://www.medicalsystems.com), was inserted via a trans-scleral, transchoroidal approach, and 1–3 μl of neural progenitor cell suspension at a concentration of 25,000–75,000 cells per μl was injected into the subretinal or vitreal space. Fellow, noninjected eyes served as one type of control. As an additional control, four newborn and four adult eyes were injected with saline (Sodium Chloride Injection BP, 0.9%; B. Braun Melsungen AG, Melsungen, Germany, http://www.bbraun.com). During and after injection, no choroidal bleeding was observed.

Histology

At 1 week, 2 weeks, and 1, 1.5, 2, 3, and 4 months postinjection, animals were sacrificed and eyes were enucleated for histological and immunohistochemical examination. After transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, eyes were embedded in paraffin and sectioned at 4-μm serial sections. Each fifth slide was stained with hematoxylin and eosin for histomorphologic evaluation.

Immunohistological Studies

Specimens were de-parafinized in xylene and dehydrated in graded alcohols, rinsed with phosphate-buffered saline (PBS, pH 7.4), and incubated with 10 mM citrate buffer (pH 6.0) at 110°C for 4 minutes. After washing with PBS, specimens were blocked for 1 hour at room temperature with PBS solution containing 1% bovine serum albumin, 0.1% triton-x100, and 3% normal goat serum. When necessary, goat serum was replaced by donkey serum in the blocking solution. Subsequently, sections were incubated for 48 hours at 4°C in a humidified chamber with one of the following primary antibodies: anti-GFP (rabbit polyclonal, 1:100; Santa Cruz Biotechnology, Inc.), anti-rhodopsin (mouse monoclonal, 1:100; Lab Vision Corporation), anti-human mitochondria (mouse monoclonal, 1:20; Lab Vision Corporation), anti–blue-sensitive opsin (goat polyclonal, 1:100; Santa Cruz Biotechnology, Inc.), anti-NRL (rabbit polyclonal, 1:2,000; generously donated by Dr. Swaroop), anti–GFAP (rabbit polyclonal, 1:100; DakoCytomation), anti–neurofilament 70 (mouse monoclonal, 1:100; Chemicon International, Inc.), anti–β-tubulin III (mouse monoclonal, 1:400; Sigma), anti-human KI-67 antigen (mouse monoclonal, 1:50; DakoCytomation), anti-synaptophysin (mouse monoclonal, 1:20; DakoCytomation), and anti–Caspase-3 (rabbit polyclonal, 1:50; Santa Cruz Biotechnology, Inc.). After washing in PBS, specimens were incubated for 1 hour at room temperature with one of the following secondary antibodies: Texas Red–conjugated goat anti-mouse IgG (1:100), Cy™2-conjugated goat anti-rabbit IgG (1:200), Cy™2-conjugated goat anti-mouse IgG (1:200), Cy™5-conjugated goat anti-rabbit IgG (1:200), and rhodamine Red™-X–conjugated donkey anti-goat IgG (1:200; all from Jackson ImmunoResearch Laboratories, Inc.). Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI)–containing mounting medium (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). To determine the specificity of the antigen–antibody reaction, corresponding negative controls with an irrelevant isotype-matched antibody were performed. A Zeiss Axiovert 200 microscope equipped with Sensi Cam 12 Bit imaging was used for fluorescent and light microscopy imaging. Confocal images were acquired using a Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 135 M inverted microscope. Channels for rhodamine, Cy2, Cy5, and UV fluorescence were used, in addition to Nomarsky optics. All double-labeled cells were analyzed at multiple consecutive planes to ensure the colocalization of nuclear, cytoplasmic, or membranal signals to the same cell.

Quantification

The number of engrafted cells was counted in a total of eight eyes (four with subretinal grafts, 8–16 weeks after transplantation and four with intravitreal grafts, 4 weeks after transplantation) using methods previously published [20]. Briefly, the area and borders of the grafts were defined by anti-GFP staining, and nuclei of hESC-derived cells were counted in sequential 4-μm sections, 60 μm apart, using a computerized image analysis system (Image-Pro version 4.1; Media Cybernetics, Silver Spring, MD, http://www.mediacy.com). Cell counts from serial sections were corrected according to the Abercrombie method (1946) and adjusted to the total size of the graft [21]. In five eyes with subretinal grafts, sections were available for counting of Rhodopsin$^+$ (Rho$^+$) cells. In each eye, four sections from the main area of the graft were randomly selected for quantification. In each section, the percentage of cells that coexpressed Rhodopsin and GFP out of the total number of engrafted cells was calculated.

RESULTS

Development of hESC-Derived NPs

To assist in the identification of transplanted cells within the retina, we used genetically modified hESCs that constitutively expressed eGFP. Stable expression of eGFP driven by the human EF1α promoter was accomplished using a lentiviral vector system, according to our published protocol [19]. Differentiation of hESCs (Fig. 1A) into highly enriched cultures of proliferating NPs was accomplished as we have previously described, with some modifications [17, 22]. Briefly, a two-step protocol was used: In the first step, hESC colonies were cultured for prolonged periods on feeders in the presence of the BMP (bone morphogenetic protein) antagonist noggin. Under these culture conditions, the hESCs in most of the colonies differentiated almost uniformly into tightly packed small progenitor cells. In the second step, clumps of approximately 150 progenitor cells

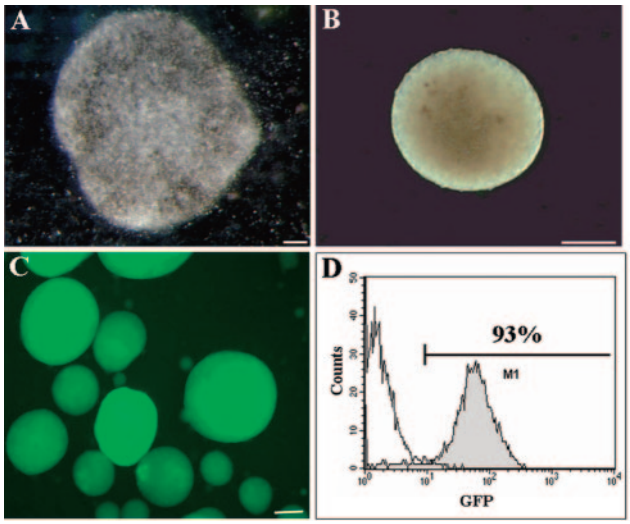

Figure 1. Derivation of neural spheres from human embryonic stem cells (hESCs) after transduction by a lentiviral vector harboring enhanced green fluorescent protein (eGFP) under the control of the human elongation factor 1 α promoter. Dark-field microscopy images of an undifferentiated hESC colony **(A)** and hESC-derived sphere **(B)**. Fluorescent image of the spheres illustrates the expression of eGFP **(C)**. Single-color flow cytometry demonstrating eGFP transgene expression by 93% of the cells within neural spheres **(D)**. (Fluorescence of control, nontransduced neural precursors is demonstrated by line, and that of transduced precursors by filled plot). Scale bars = **(A)** 100 μm, **(B, C)** 200 μm.

were mechanically isolated and replated in serum-free medium supplemented with the mitogens basic fibroblast growth factor (FGF2) and epidermal growth factor (EGF). Under these culture conditions, the clumps formed free-floating spherical structures (Figs. 1B, 1C) that were subcultured and expanded for 4 weeks [17].

Characterization of Cells Within Cultured Spheres

The phenotype of the cells within the spheres, as well as their developmental potential to give rise to retinal cells, was characterized after a 4-week propagation period. After disaggregation of the spheres, FACS (fluorescence-activated cell sorter) analysis demonstrated that 93% of the cells within the spheres expressed the eGFP transgene (Figs. 1C, 1D). Indirect immunofluorescence analysis of the expression of the neural progenitor markers PSA-NCAM, A2B5, NCAM, and nestin (Figs. 2A–2D) demonstrated that >87% of the cells within the spheres expressed these markers (Fig. 2Q; PSA-NCAM 87.3% ± 2.7%, A2B5 93.0% ± 3.0%, NCAM 87.7% ± 2.6%, nestin 98.0% ± 0.6% [mean ± SE]). Thus, at 4 weeks after derivation, the sphere cultures were highly enriched for NPs.

Successful differentiation of the hESC-derived NPs into retinal neurons probably requires the induction of the same key regulatory genes that are expressed by neural progenitor cells during the development of the retina in vivo [23–25]. Among these key genes are *Pax6*, *Rx*, *Six3*, and *Six6*, which constitute a highly conserved self-regulatory genetic network of transcription factors that are involved in the specification and morphogenesis of the eye [26–31]. Other key genes involved are the transcription factor *Lhx2*, which is essential for the transition from optic vesicle to optic cup [32], and the transcription factor *Chx10*, which regulates, at least in part, the proliferation of retinal progenitors [33]. Semiquantitative RT-PCR analysis indicated that hESC-derived NPs express these regulatory genes of retinal development, as well as genes involved in photoreceptor differentiation, including the CRX protein [34, 35], recoverin [36], NRL [37], and blue cone opsin [35]. This suggests that the NPs possess the developmental potential to generate retinal cells (Fig. 3). It should be noted that *Oct4* was not expressed by the sphere cultures, suggesting that these cultures did not contain undifferentiated hESCs.

Characterization of the NPs After Spontaneous Differentiation In Vitro

Upon withdrawal of mitogens from the medium and plating on laminin-coated culture dishes, the spheres attached rapidly, and cells migrated out to form a monolayer of differentiated cells. These cells displayed morphological characteristics and expressed structural markers of immature neurons, such as β-tubulin III, and the 70-kDa neurofilament protein (NF-L, Figs. 2E, 2F). Cells expressing β-tubulin III comprised 41.3% ± 6.4% of the total population (Fig. 2Q). Differentiated cells also expressed markers of mature neurons, such as the 160-kDa neurofilament protein (NF-M, Fig. 2G) and MAP2ab (Fig. 2H). Furthermore, the cultures contained cells that synthesized GABA, TH, serotonin, and glutamate (Figs. 2J–2M). Subtypes of neurons producing these neurotransmitters are also present within the retina in vivo [38, 39]. The NPs also showed the capacity to differentiate into glial cells, as demonstrated by the presence of differentiated cells expressing the astrocytic marker

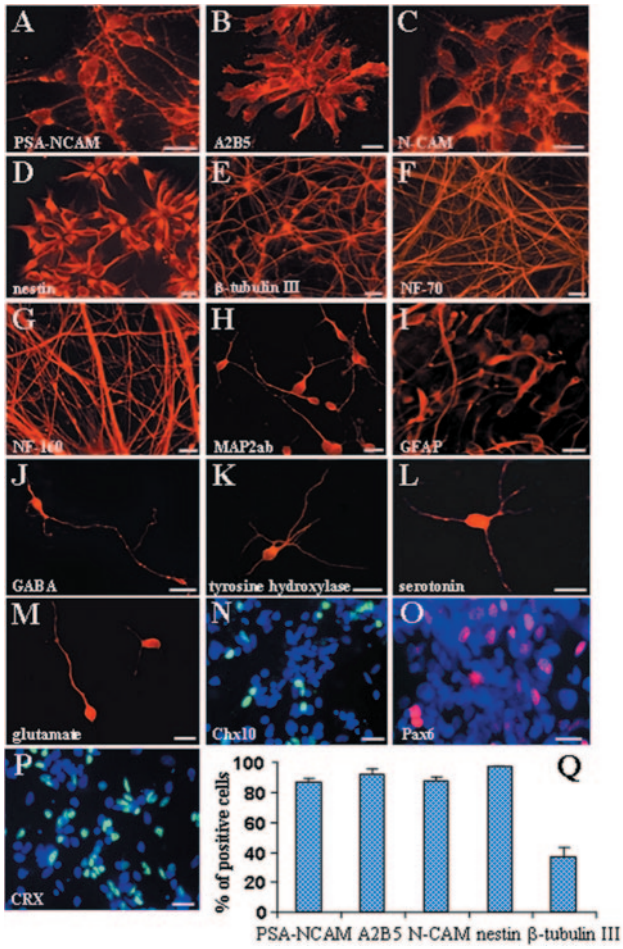

Figure 2. Characterization of cells within neural spheres. Indirect immunofluorescence staining of the precursor cells 18 hours after disaggregating the spheres and plating on adhesive substrate, for PSA-NCAM **(A)**, A2B5 **(B)**, NCAM **(C)**, and nestin **(D)**, demonstrated that >87% of the cells within the spheres expressed markers of neural precursors **(Q)**. After spontaneous differentiation, the cells acquired neuronal-like morphology and expressed markers of immature neurons, such as β-tubulin III **(E)** (41.3% ± 6.4% of the cells) and light-chain neurofilament (70 kDa) **(F)**, as well as markers associated with mature neurons, such as neurofilament 160 kDa **(G)** and MAP2ab **(H)**. Differentiated cells also expressed GABA **(J)**, tyrosine hydroxylase **(K)**, serotonin **(L)**, glutamate **(M)**, and GFAP **(I)**. Cells expressing Pax6, a marker of anterior brain and retinal differentiation, as well as ocular-specific markers expressed by proliferating retinal progenitors (Chx10) and during photoreceptor differentiation (CRX) are demonstrated in **(N–P)**. The images represent areas of the slides in which these cells were more abundant. Scale bars = 20 μm. Abbreviations: CRX, cone-rod homeobox; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; MAP2ab, microtubule-associated protein 2ab; PSA, polysialic acid.

GFAP (Fig. 2I). The expression of transcripts of regulatory genes, which govern retinal development, and those of photoreceptoral markers was maintained in the differentiated progeny (Fig. 3). Indirect immunofluorescence studies showed that 32.3% ± 2.3% of the cells expressed Pax6, a marker of anterior brain, retinal development, and postmitotic amacrine and ganglion cells (Fig. 2O) [26–28, 40]. Progeny expressing ocular-specific markers, including Chx10, which is expressed by proliferating retinal progenitors and mature bipolar cells [33], and

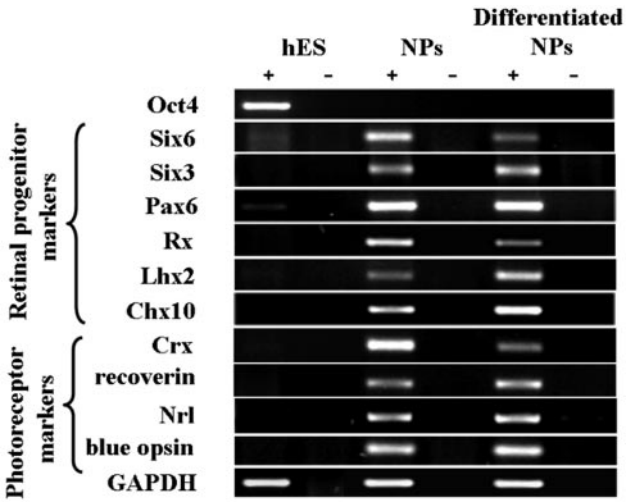

Figure 3. Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of the expression of transcripts of key regulatory genes of anterior brain and retina development as well as markers of photoreceptors within cultures of both undifferentiated and differentiated neural precursors (NPs). Human embryonic stem (hES) cell–derived NPs express key regulatory genes of anterior brain and retinal development. Transcripts of Oct4, a marker of undifferentiated hES cells, were not expressed by the NPs. The symbols + and − indicate whether the PCR reaction was done with or without the addition of RT.

CRX, which is expressed during photoreceptor differentiation [34, 35], was relatively uncommon and comprised <0.1% of the total number of differentiated cells. They were sparse throughout the slide, with limited areas where they were more abundant (Figs. 2N, 2P). Differentiated retinal cells expressing the photoreceptoral proteins NRL, blue cone opsin, and rhodopsin were not observed.

Thus, we were able to establish highly enriched cultures of hESC-derived NPs that express key genes of retinal and photoreceptor development, as well as transcripts of markers of mature photoreceptors. However, under our differentiation-inducing culture conditions, the NPs failed to express these markers at the protein level.

Survival, Incorporation, and Differentiation of the NPs After Transplantation into Rat Eyes

Given the potential of the NPs to express key regulatory genes of retinal development in vitro, we hypothesized that further differentiation of the hESC-derived NPs into retinal neurons may be brought about by inductive signals operative in the retinal microenvironment. To test this hypothesis, we examined whether the ocular microenvironment, and specifically the subretinal space, could promote further differentiation of the NPs toward a retinal fate. Single-cell suspension of NPs derived and propagated 4 weeks in culture were engrafted into the subretinal and/or vitreal space in 19 adult and 34 neonatal (2- to 3-day-old) rats. Sixty thousand to one hundred thousand NPs were engrafted per eye. The rats were sacrificed for histopathological analysis of the grafts at sequential time points between 1 and 16 weeks after transplantation. Vehicle-transplanted rats (four adult rats and four neonates) served as controls.

Localization and Migration of Grafted Cells

Indirect immunofluorescence and immunohistochemical staining with anti-GFP, human-specific anti-mitochondrial, and anti-human Ki67 confirmed the presence of human cells in 8 out of 19 (42%) transplanted adult eyes and in 15 out of 34 (44%) neonatal eyes (Figs. 4D–4L, 5B, 5C, 5E, 5F, 5H, 5I, 5K, 5M, 5O, 6D, 6I, and 6N). The residual eyes, namely 11 of 19 (58%) adult and 19 of 34 (56%) neonate eyes, were considered as failed transplantation. Human-derived cells were identified as late as 16 weeks post transplantation, the latest time point examined (Table 1). In most cases (19 of 23 successfully transplanted eyes), large main grafts were present (e.g., Figs. 4A–4C), presumably reflecting the site of injection (Table 1).

The location of the main graft was subretinal in 12 eyes (four adult and eight neonates), with a large cluster of human cells localized between the host photoreceptors and the retinal pigmented epithelium (RPE) layer (Figs. 4A, 4D, 5B, 5E, and 5H). In 10 eyes (four adult and six neonates), there were vitreal grafts with a cluster of human cells between the inner retina and the lens (Figs. 4B, 4C, 4F, 5J, 5L, and 5N). In three of these eyes (two adult, one neonate), a concomitant subretinal graft was also present. Occasionally, direct extension from the graft into the retina was observed (Fig. 4C). An isolated, small intraretinal graft was found in one additional eye (not shown), and in three eyes dispersed human cells within the inner retina were present, without a main graft being identified (Table 1).

Table 1. Summary of the age at transplantation, time interval until histopathological analysis, and the location of the graft in successfully transplanted rats

Eye no.	Age at transplantation	Time interval between transplantation and analysis (weeks)	Main graft location
1	Adult	1	IV
2	Adult	1	IV
3	Neonate	1.5	IR (small)
4	Neonate	2	SR
5	Adult	2	SR
6	Adult	2	SR+IV
7	Neonate	4	SR
8	Neonate	4	SR
9	Neonate	4	SR+IV
10	Neonate	4	IV
11	Neonate	4	IV
12	Adult	4	SR+IV
13	Adult	4	None
14	Adult	4	None
15	Neonate	8	SR
16	Neonate	8	SR
17	Neonate	8	IV
18	Neonate	8	None
19	Adult	8	SR
20	Neonate	12	SR
21	Neonate	16	SR
22	Neonate	16	IV

Abbreviations: IR, intraretinal; IV, intravitreal; None, a main graft was not seen, only dispersed human cells throughout inner retina; SR, subretinal.

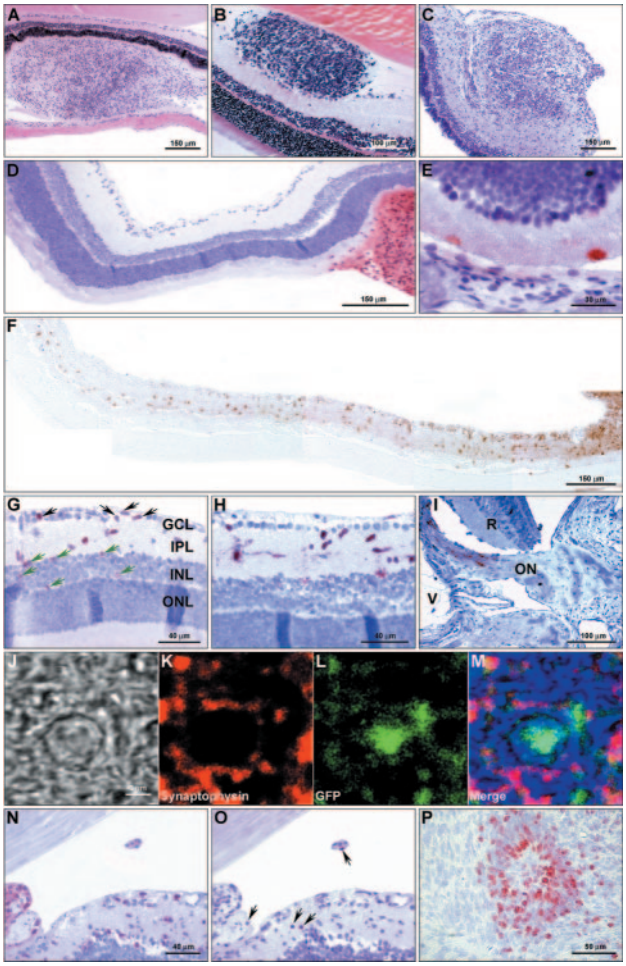

Figure 4. Histological and immunohistochemical characterization of human neural precursors after intraocular grafting. **(A–C):** Hematoxylin and eosin staining. **(D–I, N):** eGFP immunohistochemical staining. **(J–M):** Confocal images of indirect immunofluorescence staining of synaptophysin and eGFP. **(O–P):** Human-specific Ki67 immunohistochemical staining. **(A):** Subretinal graft in a neonatal rat eye 12 weeks after injection. Note that whereas the host retina is displaced by the graft at the site of injection, its general structure is maintained. **(B):** Intravitreal graft 1 week postinjection into an adult rat eye. Note grafted cells localized between inner retina and lens. **(C):** Intravitreal graft merging with inner aspect of host retina 16 weeks postinjection into a neonate eye. **(D, E):** Immunohistochemical staining with anti-eGFP showing minimal migration of transplanted $eGFP^+$ cells (brown) into the host retina **(D)**, mainly within the photoreceptor outer segment layer **(E)**, after transplantation to the subretinal region (adult eye, 4 weeks postinjection). **(F):** In contrast, wide dispersion and migration of transplanted $eGFP^+$ cells (brown), in particular in neonate-injected eyes, after intravitreal grafting. Note large number of $eGFP^+$ cells in IPL, with decreasing cell density as a function of distance from the main graft (16 weeks postinjection into neonate eye; composite of six overlapping images). **(G):** Immunohistochemical staining with anti-eGFP of an adult eye, 8 weeks postinjection, shows that in areas distant from the main intravitreal graft, dispersed transplanted cells integrate within the host ganglion cell (black arrows) and INLs (green arrows). These cells could not be separated from the host cells surrounding them based on their histological appearance alone. **(H):** Transplanted cells in the IPL (brown) extending neurite-like processes within this layer (4 weeks postinjection into neonate eye). **(I):** $eGFP^+$ processes within the ON (4 weeks postinjection into neonate eye). **(J–M):** Confocal images showing coexpression of synaptophysin and eGFP: **(J)** Nomarsky optics, **(K)** anti-synaptophysin staining (red), **(L)** anti-eGFP (green), and **(M)** merged confocal image. **(N–P):** Assessment of the proliferative state of transplanted cells. **(N):** Main intravitreal graft and transplanted cells in its vicinity, marked by eGFP immunohistochemical staining (4 weeks postinjection into neonate eye). **(O):** Adjacent section shows that only a few of the transplanted cells are $Ki67^+$ (brown). **(P):** In contrast, many $Ki67^+$ cells are present within a rosette in a subretinal graft (adult eye, 8 weeks postinjection). Scale bars = **(A, C, D, F)** 150 μm, **(B, I)** 100 μm, **(P)** 50 μm, **(G, H, N, O)** 40 μm, **(E)** 30 μm, and **(J–M)** 3 μm. Abbreviations: eGFP, enhanced green fluorescent protein; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ON, optic nerve; ONL, outer nuclear layer; R, retina; V, vitreous.

In eyes with large clusters of human-derived cells at the sites of injection, immunohistochemical analysis also demonstrated dispersed transplanted cells across the retina, singly or in small clusters, and often quite distant from the main graft (Figs. 4E–4I). There was a marked difference between eyes with subretinal grafts versus eyes with vitreal grafts in this regard. Subretinal grafts remained mostly clustered at the site of injection (Fig. 4D), and there was only a little dissemination within the host photoreceptor outer segment layer (Fig. 4E), probably via migration within the subretinal space (between the photoreceptor outer segments and the RPE). Only a small number of cells migrated from the subretinal cluster and penetrated the outer nuclear layer (ONL) to the more inner layers of the retina (not shown). In contrast, vitreal grafts were associated with wide dispersion of the transplanted cells, especially in newborn-injected eyes. In these eyes, GFP^+ cells penetrated and migrated in the retina, mainly within the inner plexiform layer (IPL, Figs. 4F–4I). At 12–16 weeks after transplantation, GFP^+ cells had migrated to parts of the IPL quite distant from the main graft (Fig. 4F). There were additional GFP^+ cells in the nerve fiber and ganglion cell layers, whereas only a few cells migrated into the inner nuclear layer (INL, Figs. 4F, 4G). Interestingly, the grafted cells that were integrated in the IPL often had GFP^+ processes extending within this layer (Fig. 4H) and were immunoreactive with anti-Synaptophysin (Figs. 4J–4M). However, it is not clear whether this implies any connectivity between the grafted cells and the host retina. Human cells and GFP^+ processes were also observed in the nerve fiber layer and the proximal part of the optic nerve (Fig. 4I).

There was marked variability in the number of engrafted cells seen in different eyes. The total number of engrafted cells within four subretinal and four intravitreal grafts was evaluated by counting in serial sections throughout the whole graft followed by adjustment for the total size of the graft. The four subretinal grafts contained a mean of 197,481 $\pm$ 62,070 cells 8–16 weeks after transplantation, and 55,611 $\pm$ 24,162 cells were found within the four intravitreal grafts 4 weeks after transplantation. Because delivery of 60,000–100,000 cells was attempted per eye, proliferation of transplanted cells obviously occurred in at least some of the eyes, as detailed below.

Proliferative State of Grafted Cells

A potential complication after transplantation of hESC-derived differentiated progeny is the formation of teratoma tumors [6]. We did not observe teratomas in any of the transplanted animals. Still, proliferation of transplanted precursors, analyzed by Ki67 expression, was observed throughout the 16-week follow-up

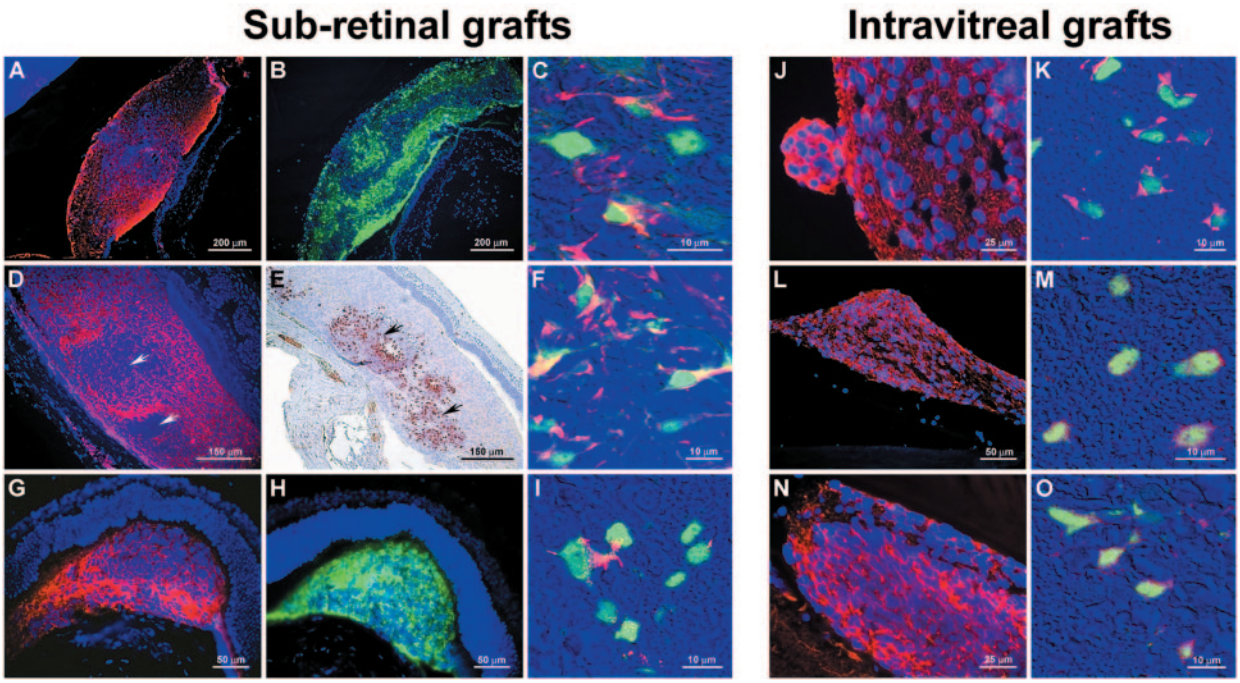

Figure 5. Transplanted human neural precursors express markers of glial, as well as neuronal, differentiation in both subretinal and intravitreal grafts. Confocal images of indirect immunofluorescence staining of three subretinal grafts demonstrating the expression of the astroglial marker GFAP **(A)** and the neuronal markers light-chain neurofilament (NF-70) **(D)** and β-tubulin III **(G)** within the grafts. The human identity of the cells within the grafts is confirmed by immunostaining for eGFP **(B, H)** (green) and for human-specific Ki67 **(E)** (immunohistochemistry, brown) in adjacent sections. Areas in which grafted cells show rosette-like formations with expression of the proliferation marker Ki67 **(E)** (black arrows) show markedly less expression of NF-70: corresponding areas in **(D)**, white arrows. This suggests a lower state of differentiation in these cells. At the single cell level, eGFP (green) is shown to colocalize with GFAP **(C)**, NF-70 **(F)**, and β-tubulin III **(I)**. The expression of the neural markers (GFAP, NF-70, and β-tubulin III) is also demonstrated within cells of intravitreal grafts in frames **(J, L, N)**, respectively (three independent grafts). Numerous cells within these intravitreal grafts also expressed eGFP and human-specific mitochondrial antigen (not shown). The coexpression of the neural markers and eGFP by single cells is illustrated in the high-magnification confocal images **(K–O)**. **(A, B, D, G, H, J, L, N):** (Blue) DAPI counterstaining. Scale bars = **(A, B)** 200 μm, **(D, E)** 150 μm, **(G, H, L)** 50 μm, **(J, N)** 25 μm, and **(C, F, I, K, M, O)** 10 μm. Abbreviations: DAPI, 4, 6-diamidino-2-phenylindole; eGFP, enhanced green fluorescent protein; GFAP, glial fibrillary acidic protein.

period (Figs. 4O, 4P, 5E). Occasionally, the NPs formed rosette structures (Figs. 4P, 5E). The percentage of Ki67$^+$ cells out of the total number of grafted cells within a cluster or layer was determined by counting the number of GFP$^+$ grafted cells in an adjacent (4 μm–apart) section (e.g., Figs. 4N, 4O) or by counting the total number of nuclei within morphologically defined grafts (e.g., Fig. 5E). Whereas 46.8% $\pm$ 2.3% (mean $\pm$ SE) of the NPs expressed Ki67 in vitro, just before transplantation, the percentage declined to 10.0% $\pm$ 2.4% in vivo ($\pm$ SEM, $n = 16$ grafts, analyzed at 2–16 weeks post transplantation). The percentage of Ki67$^+$ cells was similar in grafts analyzed 2–4 weeks after transplantation, compared with 8–16 weeks post transplantation (8.5% $\pm$ 3.1% [$n = 9$ grafts] versus 12.1% $\pm$ 3.8% [$n = 7$ grafts], respectively; $p = .47$). Cells expressing Ki67 were more abundant within large clusters of grafted cells, especially in areas with rosette-like formations, and less often seen among dispersed cells. The extent of apoptosis was evaluated within four grafts, 4–12 weeks after transplantation, by immunostaining with anti–Caspase-3. Engrafted cells expressing Caspase-3 were only rarely observed.

Fate and Differentiation of Transplanted Cells

Indirect immunofluorescence studies using cell type–specific antibodies demonstrated the differentiation of the transplanted NPs into glial and neuronal lineages within both subretinal and intravitreal grafts. Cells immunoreactive with anti-GFAP antibodies, a marker of astroglial cells, were frequently observed (Figs. 5A, 5C, 5J, and 5K). Cells that expressed the neuronal markers β-tubulin III and light-chain neurofilament (NF70) were also abundant within the grafts (Figs. 5D, 5F, 5L, 5M [NF70] and 5G, 5I, 5N, 5O [β-tubulin III]). In areas of the grafts with abundant Ki67$^+$ cells (Fig. 5D), the expression of NF70 was markedly reduced (Fig. 5E), suggesting that the cells within these areas were at a lower state of differentiation.

To test the capability of the ocular microenvironment to promote the differentiation of engrafted NPs into photoreceptor cells, we analyzed the expression of photoreceptor-specific markers by the engrafted cells (Fig. 6). Double-labeling studies showed cells that coexpressed human-specific markers and the photoreceptor markers NRL, blue cone opsin, and rhodopsin (Fig. 6) in seven eyes. It should be noted that we did not observe transplanted cells with fully formed inner and outer segments, characteristic of mature photoreceptors. Rather, rhodopsin and blue cone opsin expression was observed in the cytoplasm of round and elliptical cells (Figs. 6H, 6J, 6M, and 6O). In addition, transplanted cells expressing photoreceptor markers were infrequently observed. They were present only in subretinal

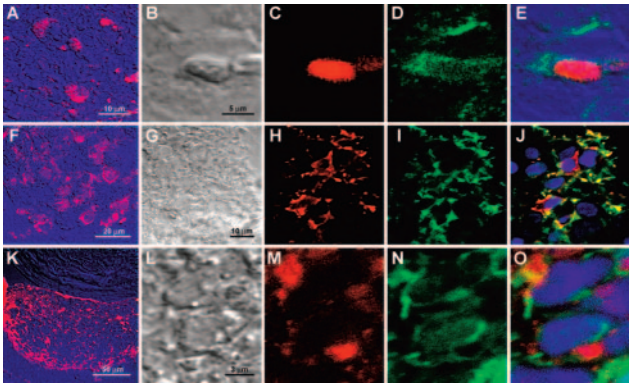

Figure 6. Transplanted human neural precursors in subretinal grafts express photoreceptor-specific markers. Cells expressing Nrl, a transcription factor involved in photoreceptor differentiation (top row, red **[A, C, E]**), blue cone opsin (middle row, red **[F, H, J]**), and the rod chromophore rhodopsin (bottom row, red **[K, M, O]**) could be identified in subretinal grafts. Groups of such cells are shown in the left column of low-power images **(A, F, K)**. In the adjacent four columns of images, double immunostaining analysis shows the coexpression of these photoreceptor markers **(C, H, M)** (red) with human-specific markers: green **(D)** anti–human-specific mitochondria staining, **(I, N)** anti–enhanced green fluorescent protein, **(B, G, L)** Nomarsky optics, and **(E, J, O)** merged confocal images; blue, DAPI (4, 6-diamidino-2-phenylindole) counterstaining. Scale bars = **(K)** 50 μm; **(F)** 20 μm, **(A, G–J)** 10 μm, **(B–E)** 5 μm, and **(L–O)** 3 μm.

grafts (7 of 11 subretinal grafts in which this was examined) and were not detected within intravitreal (nine eyes examined) or inner retinal grafts (one eye). These cells tended to appear in clusters within small regions of the grafts. In areas of Ki67$^+$ rosettes, as with the NF-70 marker, such cells were not present. The mean percentage of rhodopsin$^+$ cells out of the total number of engrafted cells was 1.47% $\pm$ 0.39% (n = 5 subretinal grafts). The presence of such cells in only the subretinal, but not in the intravitreal, grafts suggests that the subretinal compartment expressed signals and cues that were required to promote photoreceptoral differentiation of transplanted cells.

DISCUSSION

hESCs may potentially serve as an unlimited source of progenitor cells to replace degenerating retinal and RPE cells. The present study demonstrates that highly enriched cultures of NPs that express markers of anterior brain and retinal differentiation lineage could be established from hESCs in vitro. After transplantation to newborn and adult rat eyes, the NPs survived for prolonged periods, migrated, and integrated in the host retina. Differentiation into cells expressing specific markers of mature photoreceptors occurred only after subretinal transplantation, suggesting that cues in this specific microenvironment were required to support differentiation into the photoreceptoral lineage.

The concept of replacing dysfunctional or degenerated retina by transplantation has been developing ever since the first retina-to-retina transplant in 1986 [41]. In most studies, primary retinal immature (fetal) tissue has been used as donor material (for review, see [1]). It was demonstrated that such transplants can survive, differentiate, and even establish connections with the host retina to a limited degree [8, 9]. However, attempts to apply this approach clinically have not been effective thus far, mainly due to difficulties in establishing fully functional integration and perhaps have also been compounded by inflammatory or immune responses [42]. Moreover, even once these difficulties are overcome, the use of fetal retinal tissues for transplantation is severely limited by ethical constraints and practical problems in obtaining sufficient tissue supply.

An alternative approach to engrafting primary fetal retinal tissue is transplantation of NP cells. Indeed, this has recently been reported in newborn and adult normal, as well as dystrophic, animal eyes. Adult rat hippocampal progenitor cells, spinal precursor cells, as well as brain-derived precursor cell lines have been used. Survival of the cells, integration into different retinal layers, and partial differentiation into glial- and neuron-like cells were shown [10–12]. However, in contrast to our findings, differentiation toward a photoreceptor fate was not observed. Given the early embryonic origin and pluripotent nature of hESCs, it is possible that the early NPs derived from them may have a broader developmental and functional potential than the NPs derived from specific regions of adult or fetal brains, which were used in the majority of the aforementioned retinal studies. This concept is further supported by recent transplantation studies showing that mouse ESC–derived neural progeny could differentiate into functional dopaminergic neurons after transplantation to Parkinsonian rats [43–45]. This result has been largely unattainable when NPs derived from fetal or adult brains were transplanted [46–48]. Very recently, retinal progenitor cells harvested from 1-day-old mice [15] and retinal stem cells isolated from the pars plana and pars plicata in humans [16] were shown to develop into mature retinal cells (including photoreceptors), and this may provide an exciting new source for cells for retinal regeneration. Interestingly, transdifferentiation of bone marrow–derived stem cells into retinal cells expressing rhodopsin after transplantation into rat eyes, was also recently reported [13, 14]. It remains to be seen whether retinal cells transdifferentiated from non-neural stem cells will function in animal models of retinal degeneration. Additional characterization and analysis of function of putative retinal cells originating from retinal stem cells, transdifferentiation processes, ESCs, and neural stem cells are required, and it is too early in the current experimental phase to predict which type of cells will ultimately prevail in retinal transplantation therapy.

The precise cellular and molecular mechanisms that govern the development of the eye and retina are far from being completely understood. Experimental evidence in vertebrates suggests that a highly conserved self-regulatory genetic network of transcription factors plays crucial roles in morphogenesis of the eye and retinal development [23, 25, 27, 28]. The hESC-derived NPs in this study expressed many of the known key transcription factors in eye development, suggesting that they had the developmental potential to further differentiate into retinal cells.

Based on current knowledge, it appears that differentiation into the various principal cell types of the retina is probably determined by a state of intrinsic competence conferred by the transcriptional programs discussed above, that allows response to environmental cues present at the appropriate time [49]. Such extrinsic signals that augment the differentiation of progenitors to photoreceptors may include taurine [50], vasoactive intestinal peptide, retinoic acid [51], sonic hedgehog [52, 53], and activin [54]. The observation in the present study that expression of

markers of mature photoreceptors was observed only in grafted cells located in the subretinal space but not in human cells transplanted into the vitreous or allowed to differentiate spontaneously in vitro, may be related to the requirement for such site-specific extrinsic cues. The percentage of transplanted cells expressing photoreceptoral markers was low, and this may reflect lack of intrinsic competence in a majority of the transplanted cells. Further studies aimed at identifying those factors and conditions that will confer increased intrinsic competence as well as provide the necessary extrinsic cues for retinal and photoreceptoral differentiation are needed. Interestingly, differentiation of human neural stem cells into retinal cells expressing opsin was recently reported after treatment with human transforming growth factor $\beta 3$ [55].

In this study, we have shown for the first time the incorporation of hESC-derived NPs in the retina, accompanied by expression of photoreceptor-specific markers in a small percentage of the cells. Whereas our observations point out the potential of hESCs for retinal transplantation, the data also highlight key issues that need further analysis in future studies. Our data showed that delivery of transplanted cells to the appropriate location within the retina is not a trivial matter. Both in newborn and adult eyes, migration of engrafted precursors within the subretinal environment, that could promote differentiation toward a photoreceptor fate, was very limited. Whereas transplanted cells from intravitreal grafts were more extensively disseminated throughout the inner retina, the NPs migrated mainly within the IPL, and integration within the INL was sparse. Transplantation of NPs into the brain at the acute stage of stroke or in an inflammatory disease results in their migration toward the lesion and differentiation into the type of cells that were injured [56, 57]. In the eye, similar observations have been reported, with transplanted cells incorporating more readily in injured, compared with intact, retinas [58]. It remains to be seen whether degenerative processes of photoreceptors in the retina will promote a similar course with hESC-derived precursors.

Function of the grafts and connectivity with the host tissue were not assessed in the current study. Interestingly, transplanted cells, especially those dispersed in the IPL, often exhibited GFP^+ neurites and extensions in between neighboring host cells, and some also expressed synaptophysin, a synaptic marker. A few very long processes were even identified in the optic nerve exiting the eye. It is not known whether these extensions represent any functional connectivity. Clearly, this is also an issue requiring further study. The fact that transplanted ESC-derived progeny was shown to be functional in other animal models of neurodegenerative conditions is encouraging in this regard [44].

Transplantation of undifferentiated ESCs is known to be associated with teratoma tumor formation. This complication was recently reported after subretinal transplantation of mouse ESC–derived neural cells [6]. Here, the hESCs were directed to differentiate in vitro into highly enriched cultures of NPs prior to transplantation. These cultures most probably did not include undifferentiated hESCs, as suggested by the lack of expression of the transcription factor Oct4, a marker of undifferentiated ESCs. Accordingly, teratoma tumors were indeed not observed in the recipient animals. However, although a reduction in the level of proliferation of the NPs was observed after transplantation, proliferating grafted cells expressing Ki67 were still present after a prolonged follow-up of 16 weeks. This finding is in line with the observation in many eyes that the total number of engrafted cells, a few weeks postinjection, exceeded the number of cells originally transplanted. It highlights the requirement for extensive long-term studies to determine the safety of hESC-derived neural progeny transplantation.

Summary

The present study shows that hESCs can be directed in vitro to form NPs that express genes associated with retinal differentiation. Subsequently, transplantation of these cells into the local microenvironment of the subretinal space in rats allowed further differentiation and expression of specific markers of mature photoreceptors in a small percentage of cells. Whereas this subpopulation of differentiated cells is clearly not composed of fully formed photoreceptors, our findings do show that this approach is feasible and that by combining in vitro manipulation with the proper in vivo environment, differentiation toward a retinal fate can be achieved. These initial findings may be the first step toward the potential use of hESCs for future transplantation therapy of retinal degeneration.

ACKNOWLEDGMENTS

Many thanks to Dr. Anand Swaroop (University of Michigan, Ann Arbor, MI) for providing the anti-Nrl antibody, to Dr. Roderick R. McInnes (Hospital for Sick Children, University of Toronto, Ontario) for providing the anti-Chx10 antibody, to Dr. Cheryl Y. Gregory-Evans (Imperial College, London) for providing the anti-CRX antibody, to Yelena Piontek for assistance in histologic processing, and to Mark Tarshish for his help in obtaining confocal images. The study was supported by the Yedidut Research Grant and by a grant from the Israeli Ministry of Science. A.O. and M.I. contributed equally to the manuscript.

DISCLOSURES

The authors indicate no potential conflicts of interest.

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