Human Müller Glia with Stem Cell Characteristics Differentiate into Retinal Ganglion Cell (RGC) Precursors In Vitro and Partially Restore RGC Function In Vivo Following Transplantation

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INTRODUCTION

Müller cells constitute a source of retinal neurons throughout the zebrafish life [1], and several studies have highlighted the neural regenerative ability of Müller glia in the developing and postnatal retina of small vertebrate species [2]. The neural retina harbors five different types of neurons [3] that are selectively damaged during early stages of degenerative disease. Although most stem cell translational studies to repair or regenerate diseased retina have been focused on photoreceptor differentiation and transplantation of damaged retinal ganglion cells (RGCs) and their application in replacement of damaged RGCs [4], Müller glia with stem cell characteristics have been identified in the adult human eye, and although there is no evidence that they regenerate retina in vivo, they can be isolated and grown in culture and can be induced to express markers of retinal neurons in vitro [5]. Since human Müller stem cells (hMSCs) express NOTCH1 [5], and transient inhibition of NOTCH activity has been shown to induce retinal progenitors to differentiate toward a RGC fate in various experimental models [6], we examined whether a combination of growth factors and inhibition of this molecule by the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyly ester (DAPT) would induce these cells to acquire phenotypic and functional RGC phenotype in vitro and thus to provide an enriched RGC precursor population for in vivo transplantation studies. Differentiation of hMSCs into RGC precursors was confirmed by gene and protein expression analysis, changes in cytosolic [Ca2+] in response to neurotransmitters, and green fluorescent protein (GFP) expression by cells transduced with a transcriptional BRN3b-GFP reporter vector. RGC precursors transplanted onto the inner retinal surface of Lister hooded rats depleted of RGCs by N-methyl-D-aspartate (NMDA) and nicotine, and green fluorescent protein (GFP) expression by cells transduced with a transcriptional BRN3b-GFP reporter vector. RGC precursors transplanted onto the inner retinal surface of Lister hooded rats depleted of RGCs by N-methyl-D-aspartate (NMDA) and nicotine, and green fluorescent protein (GFP) expression by cells transduced with a transcriptional BRN3b-GFP reporter vector. RGC precursors transplanted onto the inner retinal surface of Lister hooded rats depleted of RGCs by N-methyl-D-aspartate (NMDA) and nicotine, and green fluorescent protein (GFP) expression by cells transduced with a transcriptional BRN3b-GFP reporter vector.
Evidence that the developmental stage of grafted cells is important for functional replacement of photoreceptors [7] suggests that the ontogenic stage of grafted RGCs may also determine whether they integrate into the retina when attempting to replace RGCs in vivo. We investigated this hypothesis using a rat model of RGC depletion induced by neurotoxic damage with NMDA [6]. In addition to the ontogenic stage of the transplanted cells, the environment into which the cells were to be transplanted was also considered in the study. Retinal degeneration is usually accompanied by microglia accumulation [8] and deposition of chondroitin sulfate proteoglycans (CSPGs) [9], both of which prevent migration and integration of transplanted cells [10]. We previously showed that migration and survival of transplanted cells can be promoted by adjuvant anti-inflammatory therapy to inhibit microglia reactivity, as well by local degradation of the extracellular matrix with chondroitinase ABC (ChABC) [10]. On this basis, we designed a strategic protocol in which hMSCs transfected with a BRN3b (a marker of RGC precursors) promoter vector were induced to differentiate into an enriched population of RGCs in vitro. These differentiated cells were transplanted onto the inner retinal surface of Lister hooded rats depleted of RGCs. The outcome of the transplantation was assessed by immunohistochemical analysis of the transplanted retina in vitro and by assessment of the RGC function by electrotetrophotography in vivo.

**MATERIALS AND METHODS**

**Isolation and Culture of Human Müller Stem Cells**

hMSC isolation and culture was performed as previously described [5]. Briefly, hMSCs were isolated from neural retina of donor human eyes using trypsin/EDTA at 37°C. The resultant cell suspension was centrifuged and the cells placed in culture on fibronectin-coated flasks in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum, and epidermal growth factor. Colonies were formed at 3–4 weeks, and confluent monolayers were obtained after a further 1–2 weeks. After this period of time, cells continued growing indefinitely. Cells for transplantation were cultured for 7 days in flasks coated with basement membrane protein (bMP) (Sigma-Aldrich, Dorset, U.K., http://www.sigmaaldrich.com) in the presence of 20 ng/ml fibroblast growth factor 2 (FGF2) (Peprotech, London, http://www.peprotech.com) and 50 μM NOTCH inhibitor DAPT (Sigma-Aldrich).

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction Analysis**

Total cellular RNA was isolated from cell pellets using the RNeasy system (Qiagen, Hilden, Germany, http://www1.qiagen.com). For the reaction, 1 μg of total RNA was reverse-transcribed in 20-μl reactions consisting of 5 mM MgCl₂, 1 mM dNTP, 1 U of RNase inhibitor, 0.75 U of avian myeloblastosis virus reverse transcriptase (Promega, Southampton, U.K., http://www.promega.com), and 25 ng/μl oligo(dT)-15 primers (GE Healthcare Europe GmbH, Munich, Germany, http://www.gelifesciences.com) in 10 mmol/l Tris/HCl buffer containing 50 mM KCl for 40 minutes at 42°C and 5 minutes at 95°C in a thermal cycler (Hybaid, Basingstoke, U.K., http://www.applegate.co.uk/all-industry/hybaid). Polymerase chain reaction (PCR) amplification was performed using primers derived from the GenBank database (sequences shown in supplemental online Table 2). Amplification was performed in a 50-μl volume by addition of 1.5 mM MgCl₂, 0.2 mM dNTP, 2 U of Taq DNA polymerase (Promega), 0.5 μM primers in 50 mM KCl, 10 mM Tris/HCl, pH 8.0. The mixture was incubated at 94°C for 5 minutes, followed by 27 cycles as follows: 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute; and one cycle of 72°C for 5 minutes. Kinetic analysis of amplified products was applied to all samples for each gene to ensure that signals derived only from the exponential phase of amplification. PCR products were analyzed by agarose gel electrophoresis (1%) containing 25 ng/ml ethidium bromide.

**Western Blotting Analysis**

Cell lysis and Western blot analysis was performed as previously described [5]. Briefly, aliquots of cell lysates containing protease inhibitors were resolved on 10% NuPAGE Bis-Tris-SDS gels (Invitrogen, Paisley, U.K., http://www.invitrogen.com) for 50 minutes at 200 V in MOPS running buffer (50 mM 3-(N-morpholino)propanesulfonic acid [MOPS], 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7; Invitrogen). Proteins were transferred to nitrocellulose membranes and blocked with 5% skim milk powder and 5% fetal bovine serum. Immunodetection was performed using various monoclonal and polyclonal antibodies as listed in supplemental online Table 1. Immunocomplexes were detected by enhanced chemiluminescence (GE Healthcare) following incubation with goat or donkey antisera against rabbit, sheep, or mouse IgG coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com). Images were analyzed and processed using a Fuji image reader LAS-1000 Pro, version 2.1 (Fujifilm UK, Bedford, U.K., http://business.fujifilm.co.uk/medical/products/film-screen-systems).

**Confocal Microscopy**

For immunocytochemistry, cells were cultured on bMP-coated (10 μg/ml) Lab-Tek glass chamber slides (Nunc, Rochester, NY, http://www.nuncbrand.com). Sections of retinal tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes and incubated for 3 hours with primary antibodies diluted in 0.5% blocking reagent (Roche, Welwyn Garden City, U.K., http://www.roche.co.uk) in PBS. Antibodies used are shown in supplemental online Table 2. Mouse IgG isotypes matching test antibodies were used as negative controls. After incubation with primary antibodies, specimens were washed in Tris-buffered saline, followed by 1 hour of incubation with Alexa-conjugated secondary antibodies (Invitrogen). Slides were washed and counterstained with 2 μg/ml 4’,6’-diamino-2-phenylindole (DAPI) for 1 minute and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Fluorescent images were recorded using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com) operating in multitrack mode for fluorescence isothiocyanate (FITC), DAPI, and Cy5 fluorochromes.

**Construction of the Transcriptional BRN3b Reporter, Electroporation, and Cell Sorting**

On the basis of published Brn3b promoter data in the mouse [11], a putative human BRN3b promoter sequence was inserted upstream of the GFP cassette in a promoterless GFP vector (pEGFP1; Clontech, Palo Alto, CA, http://www.clontech.com) to generate a transcriptional BRN3b reporter expressing GFP under control of the BRN3b promoter. To design the reporter, the University of California Santa Cruz genome browser was used to analyze the sequence upstream of the BRN3b coding sequence.
to identify a putative promoter region of the human BRN3b gene. Areas of strong conservation through species in this region were used to ascribe a 1.6-kb sequence upstream of the coding region as a putative promoter region for the gene (supplemental online Table 1). This sequence was then analyzed using the FPKM Human promoter prediction software (Softberry, Inc., Mt. Kisco, NY, http://www.softberry.com) to confirm the presence of promoter motifs in the selected region. Primers were designed for PCR amplification of the chosen region from a bacterial artificial chromosome (BAC) clone (CTD-2600K9; Invitrogen) spanning the region. The restriction sites of BglII and Sall were introduced upstream and downstream of the candidate promoter region, respectively. Primers to amplify the selected 1.6-kb genomic sequence were designed using clone-manager suite and contained the BglII and Sall restriction sites (forward: BRN3b 4,586 BglII [TACCAGATCTCAACCCGGGGAAAG-TATAG]; reverse: BRN3b 6,231 Sall [AATTGTGAC-GCGTGTCCG-TAGAGGCGACTCA]). This region was then amplified by PCR from a BAC clone (CTD-2600K9; Invitrogen), digested, and inserted into the multiple cloning site of the pEGFP1 vector using T4 DNA ligase (Roche). Following transformation, clones with the correct insert as determined by gene sequencing were expanded and DNA isolated using the nucleobond AX500 maxiprep kit (Macherey-Nagel Nucleobond; Fisher Scientific, Loughborough, U.K., http://www.fisher.co.uk). Cells were electroporated in freshly prepared electroporation buffer (containing 9.45 ml of sterile PBS with 0.55 ml of 100 mM glucose and 10 μl of 1 M CaCl2) at a final cell concentration of 10^6 cells per milliliter and reporter DNA concentration of 10 μg/ml using a single pulse imparted by the Bio-Rad (Hercules, CA, http://www.bio-rad.com) Gene Pulser II at 1.2 kV and 50 μF. Transfected cells were subjected to G418 selection before culture under control and differentiating conditions. Fluorescence-activated cell sorting (FACS) (for reporter validation) and analysis were performed using the FACSCalibur.

**Neurite Analysis**

Phase contrast images of cells in each culture condition (five different fields) were acquired. The NeuronJ application of NIH ImageJ software was then applied to these images to measure the total number and length of the neurites in each field and presented relative to the total number of cells in each field. The results from three independent experiments from each of three separately isolated populations of hMSCs were collated and analyzed using Prism statistical analysis software (GraphPad Software, Inc., San Diego, http://www.graphpad.com).

**Calcium Imaging**

Cells cultured on coverslip-bottomed eight-well Lab-Tek chamberslides were loaded with the calcium indicator dye Fura Red (Invitrogen) at 2 μM in serum-free DMEM at 37°C for 30 minutes. Cells were subsequently recovered in DMEM with 10% serum at 37°C for a further 30 minutes prior to imaging in optically clear L-15 buffering medium (Invitrogen). Time lapse live cell imaging at 37°C was performed using an Olympus IX81 microscope (Olympus, Tokyo, http://www.olympus-global.com), ×40 oil immersion objective, FITC 510/16 excitation filter, and 610/40 emission filter. Cells were stimulated with 2 mM nicotine (Sigma-Aldrich) (dose based on previous calcium imaging studies with NMDA in stem cells) [12]. Ten 1-second recordings were collected before stimulation as a baseline followed by one hundred twenty 0.5-second recordings poststimulation. Every cell in the field was identified as a region of interest (ROI), and at least three different experiments (each with 10–20 ROIs) were performed per sample to estimate response kinetics and population behavior. The graphs are represented as an inverse change in the mean percentage of fluorescence since Fura Red undergoes a decline in fluorescence intensity upon calcium binding. Cell^2R software was used to analyze the kinetic responses. The calcium indicator dye Fura Red was used alone in a nonratiometric manner in these studies in contrast to the conventional use of a second dye to generate ratiometric data. As the response was not calibrated by application of a calcium ionophore, the results do not indicate the absolute values of cytosolic calcium but rather give an indication of the relative levels. To determine the specificity of the assay, the nicotine responsiveness was abolished by addition of the nicotinic receptor antagonist methyl-lycaconitine (MLA) (100 nM, n = 3) before stimulation with nicotine.

**Transplantation of RGC Precursors**

Animals were maintained according to the U.K. Home Office regulations for the care and use of laboratory animals (Scientific Procedures Act 1986). Lister hooded rats were used in the study. All animals were tolerized at birth by an intraperitoneal injection of 1 × 10^6 hMSCs using established protocols [5, 10]. Three- to 4-week-old animals used in the experiments were anesthetized; immunosuppressed with oral cyclosporine A, prednisolone, and azathioprine; and prepared for transplantation as described before [5, 10]. Injections were performed under direct visualization of the fundus through the pharmacologically dilated pupil using an operating microscope. The dorsolateral conjunctiva was opened out with a single cut followed by blunt dissection, and a 33-gauge metal needle attached to a glass Hamilton syringe containing the materials to be injected was inserted into the intravitreal space adjacent to the inner retinal surface (avoiding injury to the lens). RGC damage was induced by injection of 2 μl of NMDA (80 μM) combined with trichloroacetic acid (TCA) (80 mg/ml) in a single eye. One week after NMDA/TCA treatment, animals were injected into the same eye with 2 μl of cell suspensions containing either RGC precursors or undifferentiated hMSCs (1 μl of 4 × 10^6 cells per microliter) combined with chondroitinase ABC (1 μl of 0.01 unit/μl ChABC) onto the inner retinal surface. Four weeks following transplantation, the animals were subjected to ERGs and subsequently euthanized by terminal anesthesia and intracardiac paraformaldehyde (4%) perfusion for histological analysis.

**Electroretinograms**

Animals were dark adapted overnight for electroretinography. Rats were anesthetized, and the eye to be examined was treated with proxymetacaine drops (local anesthetic), phenylephrine drops (pupil dilator), and hyppromellose solution (for corneal hydration). Responses from one single eye were recorded at a time while the other eye was shielded to prevent loss of dark adaptation. Rats were placed into a light-tight electrically shielded recording chamber on a heat mat at 37°C, and their heads were immobilized using a stabilizing metal bar. A platinum wire loop electrode (2 mm diameter) was used to record responses to flash stimuli (3-microsecond to 10-millisecond duration; repetition rate, 0.67–0.2 Hz) presented via an LED stimulator, at an intensity of −6.5 to −1 log cd second m^-2^-^ under scotopic conditions [13, 14]. The responses were measured using Observer software and analyzed using Reviewer (Breakpoint Pty. Ltd., Glen Iris, Victoria, Australia, http://www.bps.com) and Prism statistical analysis software (GraphPad Software, Inc.).
RESULTS

Acquisition of RGC Phenotype by hMSCs In Vitro

Culture of hMSCs on bMP with FGF2 for 48 hours caused a decrease in the expression of the intracellular domain (ICD) (active form) of NOTCH protein compared with control cells cultured in the absence of this factor (Fig. 1A). Culture with FGF2 and 50 μM DAPT (the concentration that caused the maximum decline in NOTCH ICD levels without inducing cytotoxicity; supplemental online Fig. 1A) caused a further downregulation of NOTCH ICD. This effect was seen as early as 6 hours, with a subsequent decline observed at 24 and 48 hours of culture (Fig. 1A). Downregulation of NOTCH ICD protein was accompanied by a marked increase in BRN3b protein expression, which identifies committed postmitotic RGC precursors [15, 16] (Fig. 1A). Upregulation of BRN3b protein accompanied by decrease of active NOTCH was consistent across three different hMSC lines examined (supplemental online Fig. 1B). Reduction in NOTCH protein expression was confirmed by a decrease in the intensity of immunostaining of FGF2+DAPT-treated cells for both the full-length and the intracellular domain of NOTCH (Fig. 1B). Antibodies to insulin gene enhancer protein (ISL1) and Hu antigen D protein (HuD), which characteristically stain the RGC layer and cells of the inner nuclear layer in the human and rat retina (supplemental online Fig. 2), weakly stained hMSCs cultured on bMP in the presence of FGF2, indicating a weak upregulation of these proteins by this factor alone (Fig. 1C, 1D). Addition of DAPT to the culture medium significantly increased the intensity of staining and the number of ISL1-positive cells, a marker of postmitotic RGC precursors [17] (p = .0003) (Fig. 1C), as well as the number of cells staining for HUD, a marker of RGCs [18, 19] (p = .0044) (Fig. 1D). Culture with FGF2 and DAPT for 5 days also induced a marked decrease in the number of proliferating cells, as determined by immunostaining for the proliferating antigen Ki67 (p = .0048) (Fig. 1E), suggesting that cells differentiating toward the RGC phenotype were exiting the cell cycle. In addition, and as previously shown [5], cells treated with FGF2 for 7 days showed a decrease in the expression of the Müller glia markers CRALBP and Vimentin while upregulating their expression of neuronal markers (supplemental online Fig. 1C, 1D).

A large proportion of the FGF2/DAPT-treated cells showed a distinct neural morphology, with long neurites and small phase bright cell soma characteristic of neurons (Fig. 2A–2C). An objective measure of these morphological changes was performed by analyzing the length and numbers of neurites formed by each differentiated cell when cultured in the presence or absence of FGF alone or FGF2 combined with DAPT. An increase in the number (p = .03, Fig. 2D) and length (p = .02, Fig. 2E) of neurites was observed in cells cultured with FGF2 alone compared with untreated cells. However, a much greater and significant increase in the number (p = .0041, Fig. 2D) and length (p = .0367, Fig. 2E) of neurites was seen in cells treated with FGF2+DAPT.

Since BRN3b is a well-established marker of committed RGC precursors [20, 21], we also used this marker to identify the differentiation of hMSCs into RGC precursors following NOTCH inhibition by DAPT. On the basis of evidence that a sequence immediately upstream of the BRN3b coding region contains regulatory elements that influence BRN3b expression in the mouse eye [11], we identified a putative human BRN3b promoter region (1.6 kb of the sequence upstream of the BRN3b coding region) to design a BRN3b reporter construct using this sequence. A GFP coding sequence was put under the influence of this human BRN3b promoter region. Details of the methodology and sequence used to design this promoter are shown in supplemental online Figure 3, supplemental online Table 1, and the Methods section. To ensure that the reporter identified BRN3b-positive cells, the GFP+ population generated by transfection with the reporter was isolated by FACS and examined for the expression of BRN3b mRNA. Specificity of the reporter was determined by lack of BRN3b expression in human trabecular meshwork fibroblasts cotransfected with the BRN3b-GFP construct and a control pmCherry-N1 vector (supplemental online Fig. 3).

Following transfection with the BRN3b-GFP reporter vector, cells cultured with FGF2+DAPT that expressed green fluorescent protein characteristically acquired neural-like morphology (Fig. 3A, 3B). FACS of GFP+ cells showed that these cells expressed significantly higher levels of mRNA coding for BRN3b than GFP− cells (p = .0029, Fig. 3C). BRN3b/GFP+ cells also showed enrichment of ISL1 expression, with 67% of the cells showing nuclear positivity for ISL1 compared with 36% in the BRN3b/GFP− population (p = .0008, Fig. 3D). Of the GFP+ cells, only 7% were positive for the proliferation marker Ki67 compared with 47% of the BRN3b/GFP− cells (Fig. 3E, p < .0001), suggesting that hMSCs expressing BRN3b had exited the cell cycle.

RGC Precursors Derived from hMSCs Exhibit RGC Function In Vitro

Response to the glutamate agonist NMDA is commonly used to assess the acquisition of a functional neural phenotype by stem cells in vitro [12, 22]; however, Müller glia possess functional NMDA [23] receptors and respond to NMDA stimulation with a rise in cytosolic [Ca2+] [23, 24]. With the exception of chick embryonic Müller cells in which mRNA but not functional expression of the β2 nicotinic receptor has been shown [25], adult Müller glia are not known to respond to nicotinic stimulation [26–28]. Since nicotine is well recognized to activate receptors on RGCs but not Müller glia [29, 30], we investigated the response of hMSCs to this agonist using calcium imaging. Our experiments revealed that upon stimulation with nicotine, undifferentiated hMSCs show negligible change in their cytosolic [Ca2+] [Fig. 4A, 4B]. Conversely, cells cultured on bMP with FGF2+DAPT, which induced acquisition of phenotypic characteristics of RGCs, showed a rapid rise in cytosolic [Ca2+] [Fig. 4A, 4B]. This response was evident within 2 seconds of stimulation and peaked by 5 seconds (Fig. 4B). Cumulative response curves (>40 cells per sample) confirmed that nicotine-induced changes in cytosolic [Ca2+] of differentiated cells were significantly higher than those observed in control cells (Fig. 4B). The specificity of this response was confirmed by blocking the nicotine receptors with 100 μM MLA, a selective αv and α6β2 nicotinic receptor antagonist [31] (Fig. 4B). Figure 4C illustrates the microscopic changes in cytosolic [Ca2+] of differentiated cells. Comparison of whole-cell population responses revealed that FGF2+DAPT treatment caused an increase in nicotine responsiveness from 27% in control cells to 97% in differentiated cells (Fig. 4D).

Migration of Transplanted RGC Precursors into the RGC Layer

We investigated whether hMSC-derived RGC precursors expressing BRN3b-GFP were able to migrate into the RGC layer
Figure 1. Notch inhibition promotes differentiation of human Müller stem cells (hMSCs) into retinal ganglion cell precursors in vitro. (A): Western blot showing that decreases in the levels of active NOTCH (ICD) are accompanied by increases in BRN3b protein expression in hMSCs cultured with FGF2 alone or combined with DAPT. (B): hMSCs cultured with FGF2 + DAPT showed a decrease in the intensity of staining for the full-length (FL) and active forms (ICD) of NOTCH as compared with control cells. (C, D): Cells cultured with FGF2 + DAPT not only showed enhanced staining for ISL1 and HUD as compared with cells cultured on matrix alone, but also the proportion of cells staining for these markers was significantly increased as shown by histograms (ISL1: *, p = .0003; HUD: ***, p = .0044, vs. FGF2 alone). (E): hMSCs cultured with FGF2 + DAPT showed a decrease in the number of cells staining for the proliferating antigen Ki67 (***, p = .0048; n = 5). Abbreviations: DAPT, N-[N-(3,5-difluorophenylacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; FGF, fibroblast growth factor; FL, full-length; HUD, Hu antigen D protein; ICD, intracellular domain; ISL, insulin gene enhancer protein.
using a rat model of NMDA-mediated RGC depletion. RGC death was induced in 3–4-week-old Lister hooded rats by intravitreal injection of 80 μM NMDA [32, 33]. One week later, hMSCs transfected with the BRN3b-GFP reporter and induced to differentiate into RGC precursors were transplanted onto the inner retinal surface. To avoid xenograft rejection, rats were immune-tolerized to hMSCs at birth by intraperitoneal injection of undifferentiated hMSCs [34]. In addition, they received oral immunosuppression (cyclosporine A, prednisolone, and azathioprine) from 2 days prior to transplantation until the end of the experiment [5, 10]. As previously described by our group, survival and migration of transplanted cells was promoted by induction of local degradation of extracellular matrix with chondroitinase ABC and by intravitreal administration of triamcinolone [10].

Histological examination of the retina at 4 weeks post-transplantation revealed that cells expressing BRN3b-GFP+ were aligned onto cells of the retinal ganglion cell layer, where they were extending long cytoplasmic projections onto each other and into the RGC layer, resembling nerve fibers (Fig. 5A). Some of the transplanted cells that extended processes into the RGC layer costained for BRN3b-GFP+ and synaptophysin, suggesting that they were forming synapses with the host cells (Fig. 5A, inset). Despite the formation of nerve fiber-like projections, BRN3b-GFP+ cells were confined to the transplanted region, and no axons were observed migrating long distances toward the optic nerve. In several sections, the nuclei of the human transplanted cells, as judged by their characteristic large size, were observed in close proximity to the apoptotic nuclei in the host’s RGC layer (Fig. 5B), suggesting that they had integrated into the host retina. More important, transplanted BRN3b-GFP+ cells that localized to the RGC layer also expressed the RGC marker ISL1 (Fig. 5C) and the neurofilament protein RT97 (Fig. 5D). Of 10 eyes examined, 8 were found to have good transplant cell survival and alignment with the host RGCs at 4 weeks after transplantation.

Transplantation of RGC Precursors Induces Partial Restoration of RGC Function in Eyes Depleted of RGCs by NMDA

To assess whether transplanted RGC precursors were able to repair RGC function, animals were subjected to ERGs 4 weeks after transplantation for examination of their a- and b-waves and scotopic threshold responses (STRs). The a- and b-waves of the ERG are thought to arise from the activity of photoreceptors and bipolar cells, whereas the STR has been established as an indicator of RGC function in rat full-field ERGs [14] and has been used to assess loss of RGC function in other models of glaucoma [13]. At high stimulus intensities (−3 to −1 log cd m⁻²), control eyes showed a characteristic initial negative a-wave followed by a positive b-wave, whereas at lower stimulus levels (<−4 log cd m⁻²) a small positive wave (forming the positive STR [pSTR]) was generated. This was followed by the generation of a negative wave (forming the negative STR [nSTR]) (Fig. 5A–5C). At the same light intensities, eyes treated with NMDA/TA showed significantly diminished a- and b-waves and pSTR, whereas the nSTR was completely eliminated. However, in eyes treated with NMDA/TA followed by transplantation of RGC precursors, a significant recovery of the nSTR was observed. This was accompanied by a partial recovery of the a and b waves of the ERG. Quantitative analysis of the nSTR revealed a statistically significant...
recovery of the nSTR amplitude in the transplanted eyes compared with the NMDA + TA-treated but not transplanted eyes ($p = .0214$ by t test, $n = 10$) (Fig. 6B). Although a slight recovery of the nSTR response was observed in eyes treated with NMDA + TA and transplanted with undifferentiated hMSCs, this response was not significant and could be attributed to the release of neuroprotective factors released by Müller glia (supplemental online Fig. 4). These data suggest that improvement of RGC function may be induced by transplantation of cells expressing RGC precursor phenotype but not undifferentiated hMSCs.

Figure 3. BRN3b/GFP reporter-positive cells exhibited retinal ganglion cell (RGC) precursor phenotype in vitro. (A): Colony of human Müller stem cells (hMSCs) transfected with the BRN3b-GFP reporter vector and cultured with fibroblast growth factor 2 (FGF2) + DAPT. (B): Individual hMSCs cultured with FGF2 + DAPT for 7 days showing a striking neural morphology with formation of long and ramified neurites. (C): Reverse transcription-polymerase chain reaction analysis of FACS of cells cultured with FGF2 + DAPT into GFP + and GFP − cells showed significantly higher levels of BRN3b mRNA expression in BRN3b/GFP + cells than in BRN3b/GFP − cells ($*, p = .0029; n = 4$). (D): hMSCs transfected with the BRN3b-GFP reporter and cultured with FGF2 + DAPT, costained for ISL1 (red) and GFP. Histogram shows that BRN3b/GFP + cells had a significantly higher number of cells expressing nuclear ISL1 ($***, p = .0008; n = 4$) than BRN3b/GFP − cells. (E): FACS-sorted hMSCs cultured with FGF2 + DAPT showed a significantly lower number of Ki67-positive cells in the BRN3b/GFP + population ($***, p < .001$) than in the BRN3b/GFP − population. Abbreviations: DAPI, 4′,6′-diamino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; GFP, green fluorescent protein.
DISCUSSION

Activation of Notch signaling plays a dual role in maintaining the neural progenitor population during the embryonic and adult life of several species [6, 35]. By inducing the expression of transcriptional repressor genes such as Hes1, NOTCH represses proneural gene expression and maintains the neural stem/progenitor cell population [35], whereas by promoting upregulation of cyclin D1 and activation of CDK2, it induces cells to enter the S-phase of the cell cycle and proliferate [36]. The present observations that NOTCH inhibition induces a decrease in hMSC proliferation while inducing their acquisition of RGC phenotype (i.e., expression of ISL1, BRN3b, and HUD) suggest that this factor plays an important role in the progenicity and proliferative ability of adult hMSCs. Although the role of NOTCH in RGC differentiation has been previously documented in the embryonic chick [37, 38] and rat retina [39], no previous studies have investigated the role of this factor in adult hMSCs. Unlike pigmented ciliary epithelium, which shows aberrant expression of neural markers without losing its epithelial phenotype in vitro [40], hMSCs that acquire neural cell markers in vitro show downregulation in the expression of Müller glial markers, such as CRALBP [5]. The present findings that downregulation of NOTCH activity induces neural differentiation of hMSCs into RGC precursors suggest that these cells are not merely Müller glia that exhibit aberrant expression of neural markers but that they truly undergo neural differentiation in vitro. It therefore can be proposed that as that seen during retinal development in other species, NOTCH inhibition causes amplification of the committed RGC precursor population within the adult human Müller stem cell population in vitro.

Response to glutamate stimulation is an essential component of the Müller glial phenotype [41], and hMSCs retain this glial property in vitro [24]. On this basis, NMDA responsiveness
could not be used as a measure of the functional maturity of the hMSC-derived RGC precursors. Our \([\text{Ca}^{2+}]\) imaging studies, however, indicate that FGF2 and NOTCH downregulation induces hMSCs to acquire RGC nicotine responsiveness. Interestingly, the kinetics of nicotine responsiveness observed in differentiated hMSCs, that is, slow recovery of cytosolic \([\text{Ca}^{2+}]\) (Fig. 6B), was similar to that previously observed in early cortical neurons [42]. Furthermore, in the embryonic retina, committed RGC precursors begin to demonstrate nicotine responsiveness before achieving complete maturation [43]. From these observations, it is possible to suggest that the differentiated cells constitute late RGC precursors and that further differentiation may be necessary for these cells to become fully functional RGCs. Nevertheless, acquisition of nicotine responsiveness in addition to gene and protein markers of RGCs indicates that functional RGC precursors in vitro could be derived from hMSCs.

RGC disease and optic nerve transection cause a specific decline in the STR, reflecting RGC function. NMDA treatment in particular has been shown to have a profound effect on the nSTR (greater than that seen on the pSTR) [14]. In this study, a significant improvement in the nSTR that had been completely abolished by NMDA was observed in animals transplanted with RGC precursors derived from hMSCs. Although some improvement in the nSTR response was observed in animals transplanted with undifferentiated hMSCs, this was not significant compared with the RGC differentiated cells. This effect could be attributed to the release of neurotrophic factors by the transplanted cells. Since differentiated RGC precursors induced a significant recovery of the nSTR, it is possible to suggest that as

**Figure 5.** Localization of retinal ganglion cell (RGC) precursors following transplantation into RGC-depleted retina. (A): Confocal projection of RGC-depleted retina 4 weeks after transplantation with human Müller stem cell-derived RGC precursors expressing the BRN3b-GFP reporter. Transplanted RGC precursors (GFP +) were observed aligning the RGC layer. In several places, GFP + cells extended neuronal-like processes into the GCL, where they costained for the synaptic protein synaptophysin (red). Side panel shows a magnification of the dotted area. Arrows show colocalization of synaptophysin with BRN3b GFP + cells (yellow). (B): Section of a transplanted rat retina stained for synaptophysin (red). Upper panel shows DAPI staining of apoptotic host nuclei (indicated by arrowheads) in close proximity to transplanted human cell nuclei, as identified by their large size (open arrows). The lower panel shows the distribution of the GFP-positive cells in relation to the host’s cells in the same section. (C): Section of a transplanted rat retina stained for the RGC marker ISL1 (magenta). Lower panel shows a magnification of the dotted area where transplanted BRN3b GFP + cells can be seen expressing the RGC marker ISL1 (white arrows). (D): Section of a transplanted rat retina stained for NF (red), showing colocalization of NF protein with the BRN3b GFP + cells (white arrows). Magnification of the dotted area to show detailed colocalization of NF and GFP is presented in the side panel. Scale bars = 20 μm. Abbreviations: DAPI, 4’,6´-diamino-2-phenylindole; GCL, ganglion cell layer; GFP, green fluorescent protein; INL, inner nuclear layer; IPL, inner plexiform layer; NF (RT97), neurofilament protein; ONL, outer nuclear layer; OPL, outer plexiform layer; SYN, synaptophysin.
that seen with photoreceptor transplantation in the mouse retina [7], the ontogenic state of the grafted retinal neurons might be important to restore RGC function by transplantation.

We aimed to promote survival and migration of the grafted cells using a combination of published protocols. We previously identified that inflammatory microglia and CSPGs form a natural barrier for the survival and migration of transplanted cells into degenerated retina [10], and because of the xenogenic nature of the transplanted cells, we combine immune tolerization at birth [34] with oral immunosuppression and local anti-inflammatory and matrix degradation therapy [10] to successfully overcome these barriers. Localization of hMSC nuclei (as judged by their size) among the apoptotic host cells in the RGC layer, as well as their co-staining for synaptophysin, strongly suggested that some degree of integration of the transplanted cells occurred.

The ERG results in conjunction with the anatomical localization of the transplanted cells support the hypothesis that the transplanted RGC precursors contributed to additional signaling within the RGC layer. The exact nature of this additional signaling is not known. In the absence of evidence of any projections from the transplanted cells to the brain, it is possible that transplanted cells may have established synapses with some of the remaining viable cells in the host retina and possibly aided interneuron communications. This is supported by recent findings that in the central nervous system.

Figure 6. Transplantation of retinal ganglion cell (RGC) precursors derived from human Müller stem cells (hMSCs) partially restore the nSTR in RGC-depleted retina. (A): Control (untreated) eyes showed a characteristic scotopic threshold response curve with positive and negative (nSTR, black curve) responses. RGC-depleted eyes without transplantation (NMDA/TA) showed a small positive scotopic threshold response (pSTR) but did not demonstrate a nSTR (blue curve). In contrast, transplanted RGC-depleted eyes showed a small pSTR accompanied by a partial but significant recovery of the nSTR when compared with the nontransplanted eyes (pink curve, $p = .0214$). (B): Scatter plots comparing the nSTR amplitudes of the controls and transplanted eyes. The amplitude of the nSTR of RGC-depleted eyes transplanted with RGC precursors was significantly lower than that of the control eyes ($*, p < .01$) but significantly higher than the nSTR observed with nontransplanted RGC-depleted retina ($**, p = .0214$). Of the 10 eyes included in the transplant group, 8 were found to have good histological transplant survival and integration, whereas 2 (red dots on the graph) did not. Note that these two data points have higher nSTR amplitudes compared with the others. Error bars = SEM. (C): Representative electroretinogram (ERG) responses of control, RGC-depleted eyes without transplantation (NMDA/TA) and transplanted RGC-depleted eyes (NMDA/TA/Transpl). Note the difference in curves at $-4 \log cd sm^{-2}$, the intensity at which STRs were measured. Abbreviations: NMDA, N-methyl-D-aspartate; nSTR, negative scotopic threshold response; TA, triamcinolone.
system, synapses can be found across the entire dendritic field and that amacrine cells can make highly specific synapses with direction-selective ganglion cells depending on the ganglion cell’s preferred direction [44]. Moreover, as shown in other models of stem cell transplantation, neurotrophic factors released by hMSCs may have also contributed to the recovery of RGC function, and this will form the basis of future investigations.

**CONCLUSION**

This study has demonstrated that hMSCs possess the ability to differentiate into functional RGC precursors in vitro. This was shown by their expression of RGC markers and increase in cytosolic [Ca$^{2+}$] in response to nicotinic stimulation. In vivo, when transplanted onto the inner retinal surface of RGC-depleted animals, in the presence of anti-inflammatory and matrix-degrading agents, these cells were able to migrate into the RGC layer and induce a partial recovery of RGC function. Although the present study did not address the repair of the optic nerve, the results suggest that differentiated RGC precursors may potentially be used to replace RGCs at early stages of RGC degeneration, when the major neural synapses to the optic nerve and the visual areas of the brain have not undergone neural remodeling. Although much research is needed to identify effective therapies to repair retina affected by RGC degeneration, these findings constitute the first evidence of recovery of visual function by transplantation of RGC precursors in eyes depleted of RGCs. The results pave the way to further investigate the application of adult human Müller stem cells in conditions where RGCs have been compromised but the optic nerve remains functional.

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**AUTHOR CONTRIBUTIONS**

S.S.: conception and design, performance of experimental work, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; B.B., H.J., M.F.J., and P.B.C.: performance of experimental work, collection and assembly of data, final approval of manuscript; S.B.: performance of experimental work, data analysis and interpretation, final approval of manuscript; P.T.K.: financial support and discussions; T.E.S.: data analysis and interpretation, final approval of manuscript; G.A.L.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

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