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Embryonic Stem Cell-Derived Neural Progenitors Incorporate into Degenerating Retina and Enhance Survival of Host Photoreceptors

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Abstract

Embryonic stem (ES) cells differentiate into all cell types of the body during development, including those of the central nervous system (CNS). After transplantation, stem cells have the potential to replace host cells lost due to injury or disease or to supply host tissues with therapeutic factors and thus provide a functional benefit. In the current study, we assessed whether mouse neuralized ES cells can incorporate into retinal tissue and prevent retinal degeneration in *mnd* mice. These mice have an inherited lysosomal storage disease characterized by retinal and CNS degeneration. Sixteen weeks after intravitreal transplantation into adult mice, donor cells had incorporated into most layers of the retina, where they resembled retinal neurons in terms of morphology, location in the retina, and expression of cell type-specific marker proteins. Presence of these donor cells was correlated with a reduction in the sizes and numbers of lysosomal storage bodies in host retinal cells. The presence of transplanted donor cells was also accompanied by enhanced survival of host retinal neurons, particularly photoreceptors. These results demonstrate that neuralized ES cells protect host neurons from degeneration and appear to replace at least some types of lost neurons.

Keywords

Embryonic stem cell; Retina; Transplantation; *mnd* mouse; Differentiation; Repair; Neuronal ceroid lipofuscinoses

Introduction

Mammalian embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst, are pluripotent, and give rise to all specialized cell types of the body [1–3]. In vitro, ES cells can be expanded indefinitely and can be engineered to secrete therapeutic factors. Consequently, ES cells are important potential tools for treatment of disease or injury.

Cultured ES cells can be induced to differentiate into unique cell types, including cells of ectodermal, mesodermal, and endodermal lineages [4–7]. Many studies have focused on

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Disclosures

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differentiation of ES cells to a neural lineage [8–10], including neural precursors [11–12] as well as more differentiated dopaminergic and serotonergic neurons [13].

The retina is an excellent model for studying stem cell transplantation into the central nervous system (CNS). The retina arises from the same embryonic origin as the brain, but the retina is more easily accessible than other parts of the CNS. Additionally, the organization of retinal neurons is well understood, allowing detailed determination of how transplanted stem cells interact with host cells.

When neural stem cells (NSCs) are transplanted into the vitreous of the eye, they can incorporate into the retina under very specific conditions [14–20]. Significant incorporation of transplanted NSCs into host retina apparently occurs only if the host retina is damaged due to injury or disease-related degeneration or if transplanted into normal retina before it is fully developed. In normal adult mammals, the inner-limiting membrane appears to act as a significant barrier against incorporation of NSCs from the vitreous into the retina [14].

The neuronal ceroid lipofuscinoses (NCLs) are the most common autosomal recessively inherited, neurodegenerative disorders of childhood, affecting the retina and entire CNS [21]. The degeneration is accompanied by autofluorescent accumulations within lysosomes of neurons as well as other cell types. In humans, symptoms include vision loss with photoreceptor degeneration, CNS degeneration accompanied by seizures, cognitive and motor decline, and premature death [22]. The *mnd* mouse is an important model for the NCLs. Neural degeneration in this model results from a defect in the murine orthologue of the NCL gene *CLN8* and exhibits a pathology similar to human NCLs [23–24].

In the present study, mouse ES cells were induced to a neural lineage and transplanted into the vitreous of *mnd* mouse eyes at an early stage of retinal degeneration. We examined whether the transplanted cells survive long-term and incorporate into the host retina and determined whether the transplanted cells differentiate within the retina. In addition, the ability of transplanted stem cells to reduce lysosomal storage body content and enhance survival of host photoreceptors was evaluated.

Materials and Methods

Cell Culture

Mouse ES cells (enhanced green fluorescent protein [EGFP]-expressing B5 ES cell line derived from 129/Sv mouse strain), kindly provided by Dr. Andras Nagy, were cultured using standard procedures [2, 8, 25]. Briefly, ES cells were maintained as undifferentiated colonies in the presence of leukemia inhibitory factor (LIF) (1,000 U/ml; Chemicon, Temecula, CA, <http://www.chemicon.com>). ES cells were induced to a neural lineage (neuralized) as embryoid bodies by incubation for 4 days in standard culture medium lacking LIF, followed by an additional 4 days in the same medium supplemented with retinoic acid (all-*trans*, 500 nM; Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>). For transplantation, embryoid bodies were dissociated using 0.25% trypsin with EDTA (8 minutes, 37°C) combined with mild trituration [9].

Animal Care and Use

The *mnd* mice (strain B6.KB2-*Cln8*^{*mnd*}/MsrJ, C57 mouse strain background) were purchased from Jackson Laboratories (Bar Harbor, ME, <http://www.jax.org>) and maintained as a breeding colony at the University of Missouri–Columbia. Five-week-old *mnd* mice were used for all transplants. Animals were anesthetized with i.p. injections of 80 mg/kg ketamine, 8 mg/kg xylazine, and 1.6 mg/kg acepromazine. Neuralized ES cells were concentrated to 30,000 cells/ μ l, and 1.5 μ l were transplanted into the vitreous of each eye

behind the lens with a 10- μ l Hamilton syringe and a 31-gauge needle. All animal experiments were approved by the University of Missouri-Columbia Animal Care and Use Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Sample Preparation for Morphological Analysis

The mice were euthanized via carbon dioxide inhalation at either 6 or 16 weeks after transplantation. The eyes were immediately enucleated and placed in a fixative consisting of 4% paraformaldehyde, 50 mM sodium cacodylate, and 8% sucrose (pH 7.4). For samples to be examined in cross-section, the corneas, irises, and lenses were removed immediately. The posterior portions of the eyes were incubated in the fixative at room temperature for 60 minutes with gentle agitation and then washed in 0.17 M sodium cacodylate buffer and equilibrated in 20% sucrose and embedded in optimal cutting temperature medium (Ted Pella Inc., Redding, CA, <http://www.tedpella.com>). Cryostat sections were cut at a thickness of 8 μ m, and to avoid counting errors, every sixth section was sampled for cell counts. Sections were taken within 250 μ m of the optic nerve.

Whole mounts of retinas were prepared to examine the distribution of donor cells after transplantation. For these preparations, the eyes were enucleated and incubated in the 4% paraformaldehyde fixative for 10 minutes. The corneas, irises, and lenses were then removed and the eyes were incubated in 0.17 M sodium cacodylate, pH 7.4, for at least 12 hours. The neural retinas were then separated from the eyecups and incubated in the fixative for an additional 2 hours. After the second fixation, the retinas were washed again in the cacodylate buffer. A series of radial cuts were then made in the retinas to enable the tissue to lie fairly flat [26]. The samples were then mounted on microscope slides in the cacodylate buffer. After data collection from whole mounts, the retinas were subsequently prepared for cryostat sectioning. The whole mounts were bisected in a randomly oriented plane with respect to the dorsal/ventral axis into approximately equal halves, and sections were obtained from one of the cut edges that passed through the center of the retina.

Immunocytochemistry

Fluorescence microscopy was used to determine the extent of EGFP-expressing donor cell incorporation into host retinas and to test for expression of neural markers. For those samples to be subjected to immunolabeling, cryostat sections of retinas were washed in 0.1 M phosphate-buffered saline (PBS) for 15 minutes and permeabilized for 1 hour at room temperature using 0.1 M PBS containing 0.3% Triton X-100 and 10% normal goat serum. Primary antibodies against neurons (β -III tubulin, 1:100; Promega, Madison, WI, <http://www.promega.com>; and NeuN, 1:10; Chemicon), astrocytes (GFAP, 1:100; Sigma-Aldrich), subclasses of ganglion cells, amacrine cells, and horizontal cells (calretinin, 1:200; Chemicon), bipolar cells (cPKC α , 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>), and photoreceptors (rhodopsin, 1:200; Chemicon) were applied overnight in 0.1 M PBS containing 0.5% Triton X-100 and 1% normal goat serum at 4°C, rinsed three to four times, and then exposed to an appropriate fluorescence-tagged goat anti-mouse secondary antibody or goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, <http://probes.in-vitrogen.com>) in 0.1 M PBS containing 0.3% Triton X-100 and 10% NGS for 3 to 4 hours at room temperature. In certain cases, nuclei were counterstained with either Hoechst 33358 (Sigma-Aldrich) or propidium iodide (ICN Biomedicals, Irvine, CA, <http://www.icnbiomed.com>).

Fluorescent images were obtained using one or both of two epifluorescent image capture systems. The first system was a BioRad Radiance 2000 Confocal System (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) interfaced with an Olympus IX70 microscope. The

software for this system was Bio-Rad Laser-sharp 2000. The second system was a Zeiss Axiophot microscope equipped for epifluorescence, and fluorescent emissions were stimulated with light from a 50-W high-pressure mercury vapor source. In the latter system, examination and photography of the specimens was performed using a $\times 40$ Plan-Neofluor objective with a 1.30 numerical aperture, a 395- to 440-nm exciter filter, an FT 460 chromatic beam splitter, and an LP 510 barrier filter. Photography using the latter system was performed with Kodak EliteChrome 100 ASA film, and the color slides were digitized for data analysis and reproduction.

Data Quantification and Statistical Analysis

Donor cell phenotypes and incorporation after intravitreal transplantation were quantified. Cryostat sections (8 μm thick) of the bisected eyecups used for all quantification studies were taken in a plane within approximately 250 μm of a plane perpendicular to the retina surface and passing through the optic nerve head. Thus, the sections contained both central and peripheral regions of the retina. For quantification of donor cell phenotypes, sections used were located approximately 48 μm apart (every sixth section was used with each section cut at 8- μm thickness). EGFP-expressing donor cells located within the neural retina were counted as possessing certain neural and/or retinal phenotypes, and the relative percentages of each phenotype were determined within each section.

For quantification of donor cell marker expression, a similar approach was followed except that retinal sections were labeled with appropriate antibodies as described above. Sections used for quantification were located approximately 48 μm apart (every sixth section was used with each section cut at 8- μm thickness). EGFP-expressing donor cells exhibiting phenotypes similar to neural and/or retinal phenotypes were examined for expression of various neural markers. The relative percentages of such donor cells expressing these neural markers were quantified for each section.

Quantification of autofluorescent storage bodies was obtained from counts in mounted frozen sections in matching regions of multiple ($n = 8$) host retinas. Matching regions of the retina were determined by the regions' distances to the center of the retina, near the optic nerve head. The two-dimensional surface areas of individual autofluorescent storage body aggregates were determined from digital images of cryostat sections using NIH Image (version 1.62). Samples with extensive donor cell incorporation were compared with sham-injected controls.

Quantification of host photoreceptor cells was achieved by counts of rhodopsin-positive cells in frozen sections of retinal tissue. Retinal sections were labeled with antibodies against rhodopsin, and nuclei were counterstained with Hoechst 33358 or propidium iodide. The total number of labeled nuclei colocalized with rhodopsin immunoreactivity in the surrounding cytoplasm was counted in each retinal section. Samples with extensive donor cell incorporation were compared with sham-injected samples.

Examination of the proximity of donor cells to the observed decrease in storage bodies as well as photoreceptor rescue was performed similarly to the experiments described above. In these experiments, counts were made from retinal sections divided in half near the optic nerve, and then regions were designated as follows. A side was designated as having donor cells present when greater than 20 incorporated donor cells were observed per 500- μm length of retina. A side was defined as donor cells absent when fewer than 2 incorporated donor cells were observed per 500- μm length of retina. Regions that contained between 2 and 20 incorporated cells were rare and were not included in our analyses.

For all quantitative morphological analyses, a minimum of three sections from each eye was analyzed from a total of eight host eyes. The value of a parameter for a particular eye was then determined as the average of the data from the individual sections from that eye. All data were analyzed using SPSS version 6.1 and presented in the text as means $\pm$ standard errors. The sample size was eight eyes for quantification of storage bodies and six eyes for quantification of host rhodopsin expression. A Student's *t*-test was used to determine statistical significance.

Results

Donor Cell Distribution in Host Retinas

Analysis of whole mounts indicated that by 16 weeks after transplantation of neuralized ES cells into the vitreous of *mnd* mice, donor cells were widely dispersed across the host retinas, although the distribution was not completely uniform (Fig. 1). Donor cells were found in most regions of the retina, often assuming neural-like morphologies. Donor cells occurred both as individual cells as well as occasional small clusters of cells associated with the host retina.

Donor Cells Incorporate into Host Retina

At 16 weeks after transplantation, the EGFP-expressing donor cells in the eye were either attached to the inner retinal surface or had migrated into the retina (Fig. 2, Table 1). Of all of the EGFP-positive donor cells present in the eye at this time, $27.3\% \pm 7.0\%$ had incorporated into the host retina. All of the remaining cells were closely associated with the vitreal surface of the retinal inner-limiting membrane. Many of the donor cells exhibited neuronal-like morphologies and possessed numerous branched processes with varicosities. In contrast, at 6 weeks after transplantation, most of the donor cells appeared attached to the inner retinal surface and relatively few had migrated into the retina ($n = 8$, data not shown).

The donor cells that incorporated into the host tissue at 16 weeks after transplantation assumed neuronal-like morphologies with numerous, fine processes (Fig. 2A; Table 1). Furthermore, in most instances, incorporated donor cells exhibited morphologies similar to specific retinal cell types. For instance, the morphologies of some donor cells were similar to those of ganglion cells (Figs. 2B–2D). Within the plexiform layers of the retina, we commonly observed processes of donor cells with extensive branching (Figs. 2E–2G). Other donor cells displayed morphologies similar to those of amacrine cells (Figs. 2H–2J), bipolar cells (Figs. 2K–2M), or horizontal cells (Figs. 2N–2P). These cells were located in retinal layers appropriate for the cellular phenotypes. Donor cells were rarely observed within the outer nuclear layer, and none acquired typical photoreceptor-like morphologies.

When equivalent numbers of neuralized stem cells were injected into the eyes of normal C57BL/6J mice, no incorporation of the transplanted cells into the host retina was observed ($n = 4$). In these animals, donor cells survived in the vitreous for at least 16 weeks after transplantation but did not adhere to the retinal surface. Most of the donor cells were present in large sheet-like aggregates in the vitreous. No evidence of retinal inflammation (e.g., invasion of macrophages or lymphocytes, edema, etc.) was observed in any of the treated eyes included in this study.

Donor Cells Express Neural and Synaptic Markers

Immunolabeling of retinal sections revealed that transplanted cells within the host tissue expressed general as well as relatively specific neural markers (Fig. 3, Table 2). By 16 weeks after transplantation, a large fraction of donor cells expressed the neuronal markers β -III tubulin (Figs. 3A–3C) or NeuN (Figs. 3D–3F). Several cells also expressed more specific

markers associated with particular retinal cell types. Calretinin is known to label a subclass of horizontal cells, amacrine cells, and ganglion cells. In the present study, we observed numerous donor cells that displayed amacrine cell-like morphologies and expressed the marker calretinin (Figs. 3G–3I). Other donor cells with bipolar cell-like morphologies and with cell bodies in the inner nuclear layer (INL) expressed the marker cPKC- α (Fig. 3J–3L). Rarely, donor cells were found adjacent to the outer nuclear layer (ONL). Although lacking characteristic photoreceptor morphologies, many of these cells expressed the photoreceptor-specific marker rhodopsin (Figs. 3M–3O). A mean number of 81 donor cells in sections from eight eyes were observed contiguous with the ONL. Of those cells, a mean percentage of $87.7\% \pm 4.3\%$ expressed rhodopsin. Many processes originating from donor cells may have made synaptic connections, based on the presence of numerous branched processes in the plexiform layers and the expression of the synaptic vesicle marker SV2 (Figs. 3P–3R). A small percentage of donor cells labeled positively for the astrocytic marker GFAP (Fig. 3S–3U). GFAP expression by donor cells was found almost exclusively among cells that remained along the inner retinal surface (adjacent to the inner-limiting membrane) and had not incorporated into the neural retina.

Donor Cells Reduce the Number and Size of Lysosomal Storage Body Aggregates

As mentioned above, the NCLs are characterized by the presence of autofluorescent lysosomal inclusion bodies within many types of cells, including retinal neurons [21]. We examined the effects of donor cell implantation on lysosomal storage content of *mnd* mouse retinas. In the presence of donor cells at 16 weeks after transplantation (Fig. 4), storage body aggregates were significantly ($p < .05$) fewer in number (2.24 ± 0.61 , Figs. 4A, 4C) and smaller in size (mean area = $0.44 \pm 0.04 \mu\text{m}^2$, Figs. 4A, 4D) compared with sham-injected control retinas (mean number = 4.87 ± 0.55 , mean area = $0.62 \pm 0.03 \mu\text{m}^2$).

Rescue of Photoreceptors in the Presence of Donor Cells

Although many studies have demonstrated the potential for stem cells to replace lost cells, the ability of donor cells to delay and/or prevent degeneration of host tissue is also an important consideration. The incorporation of donor cells within the *mnd* retina significantly reduced the extent of retinal degeneration, particularly the loss of host photoreceptors (Fig. 5). Normal, age-matched C57 mouse retina exhibits uniform layers of different cell types, including a highly structured photoreceptor layer (Fig. 5C). In the *mnd* mouse retina, a progressive loss of photoreceptors results in a substantial thinning of the photoreceptor cell layer (Fig. 5A), with peripheral regions of the retina exhibiting a more rapid loss of photoreceptor cells than the central region [27, 28]. In the present study, donor cell incorporation was found to be correlated with enhanced photoreceptor survival (Fig. 5). Photoreceptor cell densities were determined in retinas that were either sham-injected (Fig. 5A) or received donor cell implants and exhibited extensive donor cell incorporation (Fig. 5B). By 21 weeks of age, corresponding to 16 weeks after transplant, the mean density of photoreceptors in retinal samples with extensive incorporation (Figs. 5B, 5D) was significantly greater ($p < .05$) than in comparable regions of retinas that were devoid of stem cell incorporation (Figs. 5A, 5D) in both central (202.4 ± 12.3 rhodopsin-positive cells in the presence of donor cells, 128.7 ± 16.4 rhodopsin-positive cells in the absence of donor cells) and peripheral (147.0 ± 9.7 rhodopsin-positive cells in the presence of donor cells, 61.2 ± 4.3 rhodopsin-positive cells in the absence of donor cells) regions of the retina.

Despite significant rescue of host photoreceptors in the presence of donor cells, retinal degeneration was not prevented completely by donor cell implantation. Photoreceptor cell densities in age-matched, normal C57 mouse retinas were clearly greater than those of *mnd* retinas with extensive donor cell incorporation (Figs. 5B, 5C).

Although there were significant differences between transplanted samples compared with sham-injected samples, it remains important to determine the degree to which these effects are observed within the same host retina. It is possible that the effects of the donor cells are significant within a given proximity rather than throughout the entire retina. To address this question, a similar quantification approach was applied within individual retinal samples. The dispersal of EGFP-positive donor cells across the retina was often uneven, with some areas of the retina nearly devoid of donor cell incorporation (Fig. 1G). This phenomenon allows one to test the role of donor cell proximity on potential therapeutic effects. Upon comparison of regions of the same retinas containing substantial numbers of EGFP-positive donor cells (i.e., donor cells present; see Materials and Methods) and those regions essentially devoid of donor cells (i.e., donor cells absent; see Materials and Methods), mean lysosomal storage body number (1.91 ± 0.54 storage bodies in the presence of donor cells, 3.52 ± 1.01 storage bodies in the absence of donor cells) and size (mean of $0.384 \pm 0.037 \mu\text{m}^2$ in the presence of donor cells, mean of $0.495 \pm 0.031 \mu\text{m}^2$ in the absence of donor cells) were significantly ($p < .05$) lower in areas with donor cells present (Figs. 6A, 6B). Correlating with these results on storage bodies, we found that the densities of host photoreceptors in areas with donor cells present (192.6 ± 22.2 rhodopsin-positive cells) were significantly ($p < .05$) greater than with donor cells absent (150.8 ± 18.1 rhodopsin-positive cells) in regions of the same retinas (Fig. 6C).

Discussion

Our data indicate that neuralized ES cells transplanted into the eyes of host *mnd* mice exert neuroprotective effects on diseased retina, retarding both the accumulation of lysosomal storage bodies and photoreceptor degeneration. Photoreceptors are the primary retinal cell type lost in this model of the neuronal ceroid lipofuscinoses [28, 29], with a more modest reduction in inner retinal layers. The specific mechanisms underlying the neuro-protective actions of neuralized ES cells remain to be determined; however, it is likely that the donor cells provide a form of trophic support to the host retina, most notably to the photoreceptor layer.

NSCs are known to secrete a variety of growth factors [13, 30]. Secretion of specific growth factors by donor cells in vivo may mediate enhanced survival of photoreceptors in the *mnd* retina. Indeed, loss of cortical interneurons in the *mnd* mouse is reduced after exogenous administration of IGF-1 [31]. Administration of a variety of trophic factors has also been shown to inhibit retinal degeneration [32, 33]. If the neuroprotective effects of donor cell incorporation in *mnd* retina is indeed due to secretion of trophic factors by these cells, it may be possible to engineer donor cells to express increased amounts of the appropriate factors for even greater rescue of *mnd* host neurons.

The long-term neuroprotection provided by donor cells is accompanied by a reduction in the number and size of lysosomal storage body accumulations in host retinal neurons. The decrease in storage body content may contribute to the survival effects whereby incorporated ES cell-derived neural progenitors delay photoreceptor degeneration. However, a mechanism by which incorporation of the donor cells into the retina might retard or reverse storage body accumulation is not apparent. The *CLN8* gene that is defective in *mnd* mice encodes a putative transmembrane protein of unknown function. How the *CLN8* mutation leads to storage body accumulation remains to be determined. Until the function of the CLN8 protein is understood, it will be difficult to determine how donor cell incorporation into the retina alters host cell storage body content. If CLN8 is indeed an intrinsic membrane protein, it is unlikely that the normal protein is being transferred from the donor to the host cells in the retina. However, it is possible that the CLN8 protein is involved in the synthesis of a soluble factor that when present prevents storage body

formation. This soluble factor, rather than the CLN8 protein itself, may be what is transferred from the donor to the host cells to prevent storage body accumulation and cell death.

The retina is a highly organized structure derived from the primordial CNS. For replacement of lost retinal cells to take place, it is important that donor cells incorporate appropriately into the highly structured retinal layers. In recent years, studies have shown that stem cells derived from neural tissues can incorporate into the developing, diseased, and/or injured retina [14, 16, 17, 19, 34]. In many cases, cells with elaborate neural morphologies have been observed, often resembling closely the appearance of retinal neurons. Furthermore, the donor cells are commonly found in locations appropriate for the acquired retinal cell phenotype [14, 16, 19]. We have shown previously that neuralized mouse embryonic stem cells can incorporate into the retina of an unrelated mouse mutant with early-onset, rapid retinal degeneration. In the latter model, transplanted, neuralized ES cells survived long-term and exhibited limited differentiation into retinal neurons [9].

In the present study, we observe varied morphologies among transplanted cells, including both cells that remain on the retinal surface and those that integrate into the host retina. Incorporated donor cells always exhibit neural-like, including retinal cell-like, morphologies. Furthermore, many of the cells with retinal cell-like morphologies have cell bodies and processes located in layers of the host retina appropriate for the donor cell's phenotype.

Although donor cells that incorporate into the *mnd* retina appeared to differentiate into specific specialized types of retinal neurons, no donor cells exhibit the morphological features of normal photoreceptors. Assumption of photoreceptor-like properties by donor cells has rarely been observed in other model systems of stem cell implantation into the eye [14, 16]. However, in a recent study by Sakaguchi and colleagues [19], brain-derived neural progenitor cells transplanted into the developing marsupial retina did assume retinal cell-like morphologies, localize to appropriate retinal layers, and express appropriate retinal markers in vivo, including photoreceptor markers. Thus, it seems likely that signals necessary to induce donor neural progenitor cells to become photoreceptors may be present only before retinal development is completed. Alternatively, our donor cells may be unable to respond to such signals or donor cells differentiating into photoreceptors may be selectively lost, as are the host photoreceptors. On the other hand, the donor cells apparently were able to differentiate into most of the other specialized types of retinal neurons in our model, indicating that even in a fully developed retina, recruitment of progenitor cells to replace most cell types is possible. To achieve photoreceptor replacement, it will be important to identify signals that induce neural progenitors to differentiate into photoreceptors. Indeed, Dong et al. [35] reported that treatment of human NSCs with transforming growth factor- β induces the expression of photoreceptor markers in these cells after transplantation.

The interactions of donor cells with a host retina are very likely dependent on the specific properties of the donor cells. Several protocols currently exist to induce a neural fate in ES cells [7, 8, 25, 36–39], and recent studies have revealed more efficient means of deriving specific neural lineages from ES cells [40, 41]. Regardless of the induction method, these techniques all produce a heterogeneous mixture of neural cells. Likewise, most adult stem cell lines (including NSCs) consist of heterogeneous mixtures of cells, some of which may be committed to a particular neural fate. We have previously shown that most B5 ES cells express the neural progenitor marker nestin after being subjected to the induction protocol used in the present study [9], indicating that most of our donor cells retain a high degree of plasticity. Based on protein markers, there is heterogeneity in the stem cell population used

for transplantation in our experiments. Because most of these cells are immunoreactive for nestin, they would best be termed neural progenitors [11, 19].

Mouse ES cells are capable of expressing certain retinal genes in vitro after induction methods similar to those used in the current study [42]. Although the expression of a single marker does not prove the mature differentiation of a stem cell, it is a good indicator of a cell's developmental potential. Here, calretinin or cPKC α expression in transplanted cells does not necessarily indicate differentiation of ES cells into mature retinal neurons since these markers are also expressed elsewhere in the CNS; however, combined with the morphology and location of these cells in layers of the retina appropriate for the corresponding cell types, it suggests that the donor cells may become authentic retinal cells in vivo.

Although the presence of donor cells in host retina resulted in enhanced photoreceptor survival, the precise mechanism of this effect is not known. As described above, tissue-derived stem cells such as brain-derived NSCs are capable of integrating into host retinas under certain conditions [20]. It is possible that such cells, or other additional types of donor cells capable of integrating into the retina, would also exert a rescue effect on host photoreceptors. However, previous studies of somatic cells (e.g., fibroblasts and astrocytes) transplanted into nervous system models of Parkinson's disease show a lack of rescue of host cells unless the donor cells are genetically modified to overexpress various survival factors [43–45]. Regardless, the *mnd* mouse model is a good platform for assessing the efficacy of various donor cell types in treating neurodegeneration.

Our findings suggest that ES cells may prove to be an important tool to prevent or delay neural degeneration. Transplanted stem cells may in some cases be effective in replacing host cells lost due to pathology, but stem cells can also be engineered in vitro to produce or overexpress a gene product with therapeutic effects [46]. When transplanted, these cells can serve as a long-term source of this gene product in vivo [34, 47–49]. Indeed, in an environment as complex as the nervous system, it may be more beneficial in some cases to use stem cells primarily as vectors to deliver therapeutic factors rather than attempt to reconstruct elaborate synaptic pathways within the nervous system.

In summary, we show that mouse ES cells are able to differentiate into neural cells expressing distinctive markers and can survive long-term after neural induction and transplantation into the vitreous. Furthermore, these cells incorporate into most layers of the retina and assume phenotypes resembling those of retinal neurons. We conclude that this population of ES cell-derived cells contains neural progenitors with a high degree of developmental plasticity and thus are able to differentiate into several retinal-specific cell types. Whether donor cells that incorporate into the *mnd* retina are physiologically equivalent to host retinal cells remains to be determined. Finally, ES cell-derived neural progenitors reduce and/or delay progression of a neurodegenerative process. These results suggest that transplantation of neuralized ES cells may provide a viable form of therapy for a variety of inherited neurodegenerative diseases and/or injury to the CNS.

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Figure 1.

Donor cells become associated with the host retina after intravitreal transplantation. **(A–H):** Images of the whole-mount retina preparation displayed in the whole-mount montage shown in the center. The locations from which these images were taken are indicated in the montage. Many enhanced green fluorescent protein-expressing donor cells become widely dispersed across the retina and adopt neural-like morphologies. Scale bar = 200 μm .

Figure 2.

Donor cells incorporate within many retinal layers of *mnd* mice and acquire retinal cell-like morphologies. Transplanted cells expressing enhanced green fluorescent protein are found integrated within the inner layers and outer plexiform layer of the host retina and are widely distributed by 16 weeks after transplantation (A). Retinal cell-like morphologies are seen in most layers, including donor cells that resemble ganglion cells (B–D), extensive branching within the inner plexiform layers (E–G), amacrine cell-like cells (H–J), bipolar cell-like cells (K–M), and cells that appear to be horizontal cells (N–P). Scale bar = 80 μm (A) and 40 μm (B–P). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

Figure 3.

Transplanted cells express neural- and retinal-specific markers in vivo. Donor cells found throughout the retina display general and specific neural markers. **(A–F):** Transplanted cells acquire neuronal-like morphologies and express general neural markers, such as β -III tubulin **(A–C)** and NeuN **(D–F)**. **(G–L):** Transplanted cells with retinal cell-like morphologies can express specific markers appropriate for cells unique to particular layers, such as the amacrine cell marker calretinin **(G–I)** and the bipolar cell marker cPKC- α **(J–L)**. Donor cells immediately adjacent to the outer nuclear layer often express the photoreceptor marker rhodopsin **(M–O)**. Note that cells expressing specific markers possess morphologies appropriate for the corresponding retinal cell types found in these layers of the host retina, such as the bipolar-like cell in **(J)**. **(P–R):** Neurite-like processes expressing enhanced green

fluorescent protein are found largely within the plexiform layers and express the synaptic vesicle marker SV2. (S–U): The astrocyte marker GFAP was expressed by some donor cells. The GFAP expression was typically found in donor cells remaining on the vitreal surface of the retina. Note that some host reactive Müller cells expressed GFAP. The panels shown here represent merged images, i.e., images captured using filters specific for either GFP or rhodamine fluorescence. The respective images were given false colors (GFP, green and rhodamine; red) and then merged using Photoshop. Therefore, cell bodies and processes that appear yellow in the merged images exhibited fluorescence due to both enhanced GFP expression and labeling for the indicated marker. Scale bar = 40 μ m. Abbreviation: GFP, green fluorescent protein.

Figure 4.

The presence of donor cells reduces lysosomal storage bodies in host retina. Autofluorescent lysosomal storage bodies (arrowheads) are a signature feature of the neuronal ceroid lipofuscinoses. The yellow storage body autofluorescence and enhanced green fluorescent protein fluorescence are simultaneously visible when viewed with fluorescein isothiocyanate filters. **(A, B):** Lysosomal storage bodies are less abundant in *mnd* retinal samples in which donor cells are present at 16 weeks after transplantation **(A)** compared with age-matched sham-injected controls **(B)**. **(C):** The mean number of storage bodies is significantly less in samples with donor cell incorporation (Cells Present) compared with sham-injected controls (Cells Absent). **(D):** Likewise, the mean two-dimensional surface area of lysosomal storage

bodies in host retinas is significantly smaller in samples exhibiting donor cell incorporation compared with sham-injected controls. $*p < .01$ (**C, D**). Scale bar = 50 μm (**A, B**). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

Figure 5.

Donor cells rescue retinal photoreceptors from cell death. The primary cell type lost due to the degenerative process in the *mnd* retina is the photoreceptor. **(A):** In sham-injected, age-matched controls, the few photoreceptors (rhodopsin labeling is blue) remaining have fragmented outer segments and typically the retina contains only one or two layers of photoreceptor cell bodies, as revealed by nuclear labeling with propidium iodide (red). **(B):** At 16 weeks after transplantation, substantial numbers of photoreceptors with intact outer segments remain in host retinas that contain donor cells (green). **(C):** A normal, age-matched C57 retina. **(D):** Significantly more host photoreceptors (cells in ONL expressing Rhodopsin [i.e., Rhodopsin +]; nuclei of these cells were counted) survive in the presence of donor cells (Cells Present) in both central retina (Central) and peripheral retina (Peripheral) ($*p < .01$). The scale bar = 50 μm (C) and 25 μm (A, B). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

Figure 6.

Donor cells exert regional rescue effects upon host tissue. Reduction of storage body size and number, as well as rescue of host photoreceptors, was greater in close association with integrated donor cells. **(A):** Storage body number was significantly decreased in close association with donor cells within the same retinal samples. **(B):** Likewise, the mean storage body size was significantly reduced in close association with donor cells within the same retinal samples. **(C):** Rescue of host photoreceptors was significantly enhanced in close association with integrated donor cells. * $p < .05$ (A–C).

Table 1

Mean percentages of donor cells^a expressing morphologies similar to various retinal phenotypes

Morphology	No. of cells with morphology	Mean $\pm$ SE (%)^b
Ganglion cell-like	626	12.0 $\pm$ 3.7
Amacrine cell-like	891	17.1 $\pm$ 4.6
Bipolar cell-like	469	8.9 $\pm$ 2.4
Horizontal cell-like	104	2.0 $\pm$ 0.7

^a A total of 5,214 EGFP-expressing, donor cells that had incorporated into the host retina were sampled from retinal cryostat sections. Individual donor cells were determined with EGFP expression in combination with Hoechst 33358 nuclear labeling.

^b A total of 40% of the incorporated donor cells could be classified into one of the categories listed (morphology). The remaining cells could not be classified definitively.

Abbreviations: EGFP, enhanced green fluorescent protein; SE, standard error.

Table 2

Mean percentages of donor cells with a particular retinal cell-like morphology that expressed a corresponding marker^a

Morphology	Marker	Total no. of cells	No. labeled	Mean $\pm$ SE (%)
All donor cells in retina	β -III tubulin	2,714	2,253	83.0 $\pm$ 11.4
	NeuN	2,580	1,574	61.0 $\pm$ 12.1
Amacrine cell-like	Calretinin	427	167	39.1 $\pm$ 5.1
Bipolar cell-like	cPKC- α	293	178	60.8 $\pm$ 4.9
Glial cell-like or on the ILM	GFAP	746	701	94.0 $\pm$ 2.6

^aMorphology indicates donor cells expressing EGFP that were found integrated in any layer of the neural retina (all donor cells in retina), that acquired an amacrine cell-like morphology or bipolar cell-like morphology, or that were found in or directly adjacent to the ganglion cell layer (but did not possess the morphology of ganglion cells; i.e., were Glial cell-like). Total no. of cells indicates all EGFP-expressing cells with the indicated morphology. Labeled shows the numbers of EGFP-expressing cells that possessed the indicated morphology and labeled positively with antibodies specific for the designated markers.

Note that for percentage of donor cells expressing calretinin, we only quantified cells with amacrine cell-like morphologies. No donor cells that possessed ganglion cell-like or horizontal cell-like morphologies expressed calretinin.

Abbreviations: EGFP, enhanced green fluorescent protein; ILM, inner limiting membrane; SE, standard error.