The Fate of Heterotopically Grafted Neural Precursor Cells in the Normal and Dystrophic Adult Mouse Retina

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Purpose. To study the integration and differentiation of heterotopically transplanted neural precursor cells in the retina of adult mouse mutants displaying apoptotic degeneration of photoreceptor cells.

Methods. Neural precursor cells were isolated from the spinal cord of transgenic mouse embryos ubiquitously expressing enhanced green fluorescent protein. Cells were expanded in vitro and transplanted into the retina of adult wild-type and age-matched $\beta2/\beta1$ knock-in mice. $\beta2/\beta1$ knock-in mutants display apoptotic death of photoreceptor cells and were generated by placing the cDNA of the $\beta1$ subunit into the gene of the $\beta2$ subunit of Na,K-ATPase. The integration and differentiation of grafted cells in recipient retinas was studied 1 or 6 months after transplantation.

Results. Mutant retinas contained more donor-derived cells than wild-type hosts. Moreover, in mutants, donor cells integrated into deeper retinal layers. In both genotypes, grafted cells differentiated into astrocytes and oligodendrocytes. Only a few ganglion cell axons were myelinated by donor-derived oligodendrocytes 1 month after transplantation, whereas extensive myelination of the nerve fiber layer was observed 6 months after transplantation. Unequivocal evidence for differentiation of grafted cells into neurons was not obtained.

Conclusions. Heterotopically transplanted neural precursor cells are capable of integrating, surviving, and differentiating into neural cell types in normal and dystrophic retinas of adult mice. The particular environment of a pathologically altered retina facilitates integration of transplanted precursor cells. In principle, neural precursors may thus be useful to substitute for or replace dysfunctional or degenerated cell types. Results of the present study also indicate that replacement of retinal cell types is likely to require more appropriate donor cells, such as retinal precursor cells. (*Invest Ophthalmol Vis Sci.* 2001;42:3311–3319)

Inherited retinal dystrophies are a heterogeneous group of disorders characterized by progressive retinal degeneration. Effective therapeutic treatments of retinal dystrophies in humans are currently not available. However, animal experiments have shown beneficial effects of various therapeutic strategies, including gene therapy to substitute for the pathogenic gene, application of growth factors to minimize cell degeneration, or transplantation of committed cell types to replace dysfunctional or degenerated cells.[1–5]

Transplantation of neural precursor cells instead of committed neural cell types is emerging as an additional, promising approach to replace dysfunctional or degenerated cell types in the nervous system. Neural precursors are multipotential cells and give rise to neurons, astrocytes, and oligodendrocytes.[6–9] These cells display a markedly broader differentiation potential. When exposed to appropriate external stimuli, they are also capable of differentiating into diverse non-neural cell types of all germ layers.[10–12] Neural precursors have been isolated from the developing and adult brain and can be massively expanded in vitro, providing, in principle, unlimited amounts of cell material for transplantation (different from primary retinal cells). When transplanted into the developing or adult brain, they have been demonstrated to integrate extensively into the recipient tissue, to survive for extended periods, and to eventually differentiate into those cell types that are affected in the host.[13–19] Neural precursor cells have also been successfully used as cellular vehicles, to introduce therapeutic gene products into the diseased nervous tissue.[20,21]

To study the integration and differentiation of multipotent precursor cells in a dystrophic adult retina, we isolated spinal precursors from transgenic mouse embryos ubiquitously expressing enhanced green fluorescent protein (EGFP).[22] These cells were expanded in vitro in the presence of mitogens and subsequently transplanted into the retina of adult wild-type mice and mouse mutants displaying apoptotic degeneration of photoreceptor cells. As a mutant host, we used $\beta2/\beta1$ knock-in mice.[23] $\beta1$ and $\beta2$ are subunits of Na,K-ATPase, a heterodimeric ion pump additionally consisting of a catalytic α-subunit.[23,24] β-subunits play a pivotal role for the formation of functional Na,K-ATPases as exemplified, for instance, by the severe phenotype of $\beta2$-deficient mice.[25] Such mice display a variety of severe defects in the central nervous system (CNS), including massive apoptotic degeneration of photoreceptor cells, and die at the end of the third postnatal week.[25,26] To study whether the $\beta1$-subunit of Na,K-ATPase is able to functionally substitute for the absence of $\beta2$ expression in vivo, the $\beta1$ gene was inserted into the $\beta2$ gene by homologous recombination in embryonic stem cells.[23] As a result, $\beta1$ is expressed under regulatory elements of the $\beta2$ gene, whereas $\beta2$ expression is abolished. The $\beta2/\beta1$ knock-in mutants display a normal life span and an apparently normal development of the brain, with the only obvious exception of a slow and long-lasting apoptotic death of photoreceptor cells. Increased apoptotic degeneration of photoreceptor cells is already apparent in 3-week-old animals and results in a significantly decreased thickness and almost complete atrophy of the outer nuclear layer in 4- and 9-month-old mutants, respectively.[23]

To obtain information about the fate of heterotopically transplanted neural precursor cells in the normal and dystrophic mouse retina, we isolated such cells from the spinal cord of EGFP transgenic mice and transplanted them into the retinas of adult wild-type mice and $\beta2/\beta1$ knock-in mutants. Heterotopically transplanted neural precursor cells integrated into the mutant retina without disrupting the histoarchitecture of the host tissue. Quantitative investigations revealed that donor-derived cells were more numerous and more widely distributed in mutant retinas than in retinas of age-matched wild-type

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hosts. Grafted cells survived for extended periods in retinas of both genotypes and differentiated into neural cell types.

METHODS

Animals

Transgenic mice ubiquitously expressing EGFP under control of a chicken β-actin promoter[22] were used as donors for neural precursor cells. Animals were maintained on a C57BL/6J genetic background, and transgenic mice were identified by analyzing tail biopsy specimens for the presence of EGFP fluorescence. Neural precursors were transplanted into the retina of adult (6-month-old) wild-type mice ($n = 6$) and age-matched β2/β1 knock-in mutants[23] ($n = 6$). Four 2-month-old wild-type mice served as additional hosts. Recipient animals were maintained in a C57BL/6J-129/Ola genetic background, and their genotype was determined by Southern blot analysis of DNA isolated from tail specimens as described.[23] All animal experiments were approved by the local animal use committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture

Spinal cords were removed from 14-day-old EGFP transgenic mouse embryos. Isolation and cultivation of neural precursors was performed as described elsewhere.[19,27] In brief, tissue was placed into a defined, serum-free medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (both from Life Technologies, Karlsruhe, Germany) containing glucose (0.6%), glutamine (2 mM), HEPES buffer (5 mM), sodium bicarbonate (3 mM), N2-supplement (1:100; Life Technologies), epidermal growth factor (EGF; 10 ng/ml; Sigma, Deisenhofen, Germany), and basic fibroblast growth factor (FGF-2; 10 ng/ml; Sigma). The tissue was mechanically dissociated using a fire-polished Pasteur pipette, and cells were plated at a density of 200,000 cells/ml in uncoated tissue culture flasks, in the same medium. Cultures were passaged weekly, and cells from the third passage were used for transplantation experiments.

Immunocytochemical Analysis of Cultured Cells

To analyze the differentiation of spinal precursors in vitro, cells were plated on poly-L-lysine–coated glass coverslips and maintained in a culture medium without EGF and FGF-2, but containing 1% fetal calf serum (FCS). Differentiation of neural precursors was analyzed 2 hours or 7 days after plating. Cultures were fixed with 4% paraformaldehyde (PA) in phosphate-buffered saline (PBS; pH 7.3) and incubated with antibodies to cell type–specific markers. Polyclonal rabbit antibodies to nestin (kindly provided by Ron McKay, National Institutes of Health, Bethesda, MD) were used to identify undifferentiated cells, monoclonal mouse antibodies to β-tubulin type III (Sigma) to identify neurons, monoclonal mouse antibodies to glial fibrillary acidic protein (GFAP; Sigma) to identify astrocytes, and monoclonal mouse antibodies to myelin-associated glycoprotein (MAG)[28] to identify oligodendrocytes. Primary antibodies were visualized using Cy3-conjugated goat anti-mouse or goat anti-rabbit antibodies (Dianova, Hamburg, Germany).

Intraretinal Transplantation of Neural Precursor Cells

Cells were centrifuged, resuspended in Hanks' balanced salt solution (60,000 cells/μl), and placed on ice until transplantation. The β2/β1 knock-in mutants and age-matched wild-type mice were deeply anesthetized, a glass micropipette was inserted into the vitreous of the right eye, and approximately 1.5 μl of vitreous fluid was removed. Subsequently, the same volume of a cell suspension was injected. Intraretinal transplantation was achieved by gently lesioning the retina with the micropipette simultaneously with the injection of cells.[19,29] Before each injection, cells were triturated to maintain a single-cell suspension.

Determination of the Number of Donor-Derived Cells in Host Retinas

Four weeks after transplantation, animals were deeply anesthetized and fixed by perfusion with 4% PA in PBS. Eyes were quickly removed and serially sectioned with a thickness of 40 μm using a vibratome. Donor-derived cells in host retinas were identified by their EGFP fluorescence, the number of integrated cells in each retinal layer was determined, and the total number of donor-derived cells per host retina was calculated. Six animals from each genotype were analyzed. Statistical analyses of data were performed using the Mann-Whitney test.

Immunohistochemical Analysis of Host Tissue

Immunohistochemistry was performed to identify donor-derived neural cell types in the host tissue. To minimize bleaching of the EGFP fluorescence of donor cells during qualitative analysis of the tissue, we enhanced the endogenous EGFP signal by incubating the sections with polyclonal chicken antibodies to green fluorescent protein (Chemicon, Temecula, CA). Sections were simultaneously incubated with one of the following cell type–specific antibodies: monoclonal mouse antibodies to GFAP to visualize astrocytes, monoclonal mouse antibodies to MAG to visualize oligodendrocytes, and monoclonal mouse antibodies to β-tubulin type III or neurofilament (Roche Diagnostics, Mannheim, Germany) or polyclonal rabbit antibodies to protein gene product 9.5 (PGP 9.5; Biotrend, Köln, Germany) to visualize nerve cells. Primary antibodies were detected with Cy2-conjugated anti-chicken and Cy3-conjugated anti-mouse or anti-rabbit antibodies (Dianova). Analysis of sections was performed with a laser confocal microscope (LSM-510; Carl Zeiss, Oberkochen, Germany).

Neural precursors were also transplanted into the retina of 2-month-old wild-type mice. Retinas of these animals were analyzed for the presence of donor-derived oligodendrocytes and myelin 6 months after transplantation. Animals were perfusion-fixed, and the eyes were quickly removed, opened, and incubated with MAG antibodies for 24 hours at 4°C. Cy3-conjugated anti-mouse antibodies were used to visualize the primary antibodies. Retinas were flatmounted and examined with a fluorescence microscope (Axiophot; Zeiss).

Light and Electron Microscopy

Untreated β2/β1 knock-in mutants (7-month-old) and age-matched wild-type mice were used to analyze the histology of the retina by light microscopy. Animals were perfusion fixed with PBS containing 4% PA and 3% glutaraldehyde. Eyes were immediately opened and stored in the same fixative for at least 12 hours. Tissue was immersed in 2% OsO_4 for 2 hours, dehydrated in an ascending series of methanol, and embedded in Epon resin. Semithin sections were prepared from central regions of the retina and stained with methylene and toluidine blue.

Retinas from wild-type mice were prepared 6 months after transplantation to study donor-derived myelin at the light and electron microscopic levels. Small pieces of retinas with heavily myelinated nerve fiber layers were selected, immersed in 2% OsO_4, and embedded in Epon resin. Semithin sections were stained with methylene and toluidine blue. Ultrathin sections were counterstained with lead citrate and analyzed with an electron microscope (EM 10C; Zeiss).

RESULTS

Retina of Adult β2/β1 Knock-in Mutants

Spinal precursor cells were transplanted into the retina of 6-month-old wild-type mice and age-matched β2/β1 knock-in mutants, and host tissue was analyzed 1 month later. To obtain more information about the phenotype of β2/β1 knock-in retinas at the time of analysis, we performed light microscopy and GFAP immunohistochemistry on untreated mutant and wild-type retinas from 7-month-old animals. At this age, the thickness of the outer nuclear layer of β2/β1 knock-in retinas

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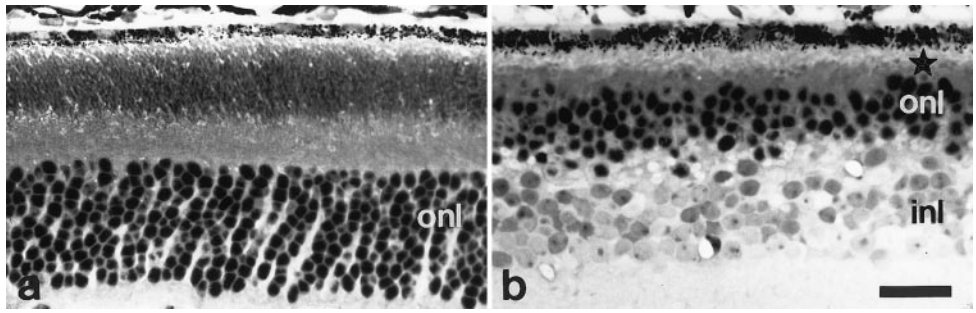

FIGURE 1. Histology of the retina of 7-month-old wild-type mice and age-matched β2/β1 knock-in mutants. The outer nuclear layer of the mutant retina showed less thickness (**b**) when compared with the wild type (**a**). β2/β1 knock-in photoreceptor cells showed short inner and outer segments (**b**, ★). Sections from both genotypes were prepared from central regions of the retina. inl, inner nuclear layer; onl, outer nuclear layer. Bar, 25 μm.

was significantly reduced when compared with wild-type retinas (compare Figs. 1a and 1b). Moreover, inner and outer segments of mutant photoreceptor cells (Fig. 1b) were significantly shorter than those of wild-type cells (Fig. 1a). Apoptotic photoreceptor cells were frequently observed in adult β2/β1 knock-in retinas, but were virtually absent from age-matched wild-type retinas (not shown).[23] Degeneration of photoreceptor cells in β2/β1 knock-in mutants correlated with elevated levels of GFAP immunoreactivity in retinal astrocytes and intense GFAP positivity of Müller cell processes (compare Figs. 2a and 2b). Neural precursor cells were thus transplanted into a pathologically altered adult retina, characterized by the apoptotic death of photoreceptor cells and reactive astrogliosis.

Differentiation of Neural Precursor Cells In Vitro

Immunocytochemical analysis of spinal precursor cell cultures 2 hours after removal of growth factors and addition of FCS identified virtually all cells as nestin positive, undifferentiated precursor cells. After a culture period of 7 days in a medium containing FCS, the majority of cells were identified as GFAP-positive astrocytes. Cultures also contained a few β-tubulin type III-positive nerve cells and MAG-positive oligodendrocytes (data not shown).

Number of Donor-Derived Cells in Adult Host Retinas

Donor cells in host retinas of both genotypes were readily identified by their expression of EGFP (Figs. 3a, 3c). In wild-type (Fig. 3a) and β2/β1 knock-in (Fig. 3c) retinas, grafted cells integrated into the host tissue without obviously disrupting the retinal histoarchitecture (Figs. 3b, 3d), and differentiated into a variety of morphologically distinct cell types. Virtually all these cells adopted a complex cytoarchitecture and extended several processes into the host retina of both genotypes (Fig. 3). In wild-type retinas, donor-derived cells were usually located in the innermost retinal layers (i.e., nerve fiber, ganglion cell, and inner plexiform layers; Fig. 3a). In mutant retinas, in comparison, EGFP-positive cells were frequently found also in deeper retinal layers (i.e., inner nuclear, outer plexiform, and outer nuclear layers; Fig. 3c). Together, transplanted spinal precursors integrated in a nondisruptive manner, differentiated into morphologically complex cell types, and survived for considerable periods (i.e., up to 6 months, see description later) in adult retinas of both genotypes.

The phenotype of the recipient retina may affect the extent and pattern of integration of grafted cells. To address this possibility, we determined the total number of EGFP-positive cells in each host retina. This analysis revealed a significantly higher number of donor-derived cells in β2/β1 knock-in retinas than in wild-type host retinas (Fig. 4a). The variability in the number of integrated cells between individual animals of each genotype (Fig. 4a) was most likely the result of variations in the transplantation procedure. Nevertheless, we consider the elevated number of EGFP-positive cells in mutant retinas to be a specific difference between host tissues of both genotypes. This view is supported by a different pattern of integration of grafted cells in β2/β1 knock-in versus wild-type retinas. Although we found similar numbers of EGFP-positive cells in the nerve fiber, ganglion cell, and inner plexiform layers of both genotypes (Fig. 4b), there was a statistically significant, genotype-related difference in the number of integrated cells in the deeper retinal layers. Both the inner nuclear and the outer plexiform layers of mutant retinas contained significantly more donor-derived cells than the corresponding layers of wild-type retinas (Fig. 4b). Moreover, some EGFP-positive cells were present in the outer nuclear layer of β2/β1 knock-in retinas. In contrast, donor-derived cells were never observed in the outer nuclear layer of wild-type recipient retinas (Fig. 4b).

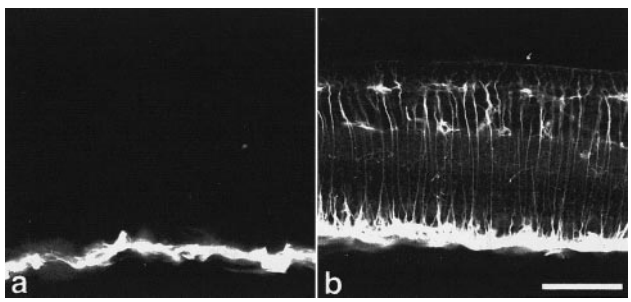

FIGURE 2. Expression of GFAP in adult wild-type and β2/β1 knock-in retinas. Expression of GFAP in the wild-type retina was restricted to astrocytes located at the vitread margin of the retina (**a**). In mutant retina (**b**), expression of GFAP was elevated in retinal astrocytes. In addition, Müller cell processes were GFAP-immunoreactive in mutant (**b**), but not in wild-type (**a**), retinas. Bar, 50 μm.

Differentiation of Neural Precursor Cells in Adult Host Retinas

Double immunohistochemistry with EGFP and cell type–specific antibodies was performed to analyze host retinas for the presence of donor-derived astrocytes, oligodendrocytes, and neurons.

Retinal astrocytes located at the vitread margin of the retina were the only GFAP-positive cells in untreated wild-type retinas (Fig. 2a). In untreated β2/β1 knock-in retinas, GFAP-immunoreactivity was additionally detectable in Müller cell processes (Fig. 2b). However, when wild-type or mutant retinas were analyzed 1 month after transplantation of neural precursors, GFAP-positive cells were detectable also in other retinal layers, and were particularly numerous in the inner plexiform layer (for a wild-type retina, see Figs. 5b and 5f). Comparison of EGFP and GFAP immunoreactivity revealed coexpression of both proteins in many if not all of these unusually positioned GFAP-positive cells and thus unequivocally identified them as donor-derived astrocytes (compare Figs. 5a and 5b or 5e and 5f; see Figs. 5c and 5g). Donor-derived astrocytes (i.e., cells posi-

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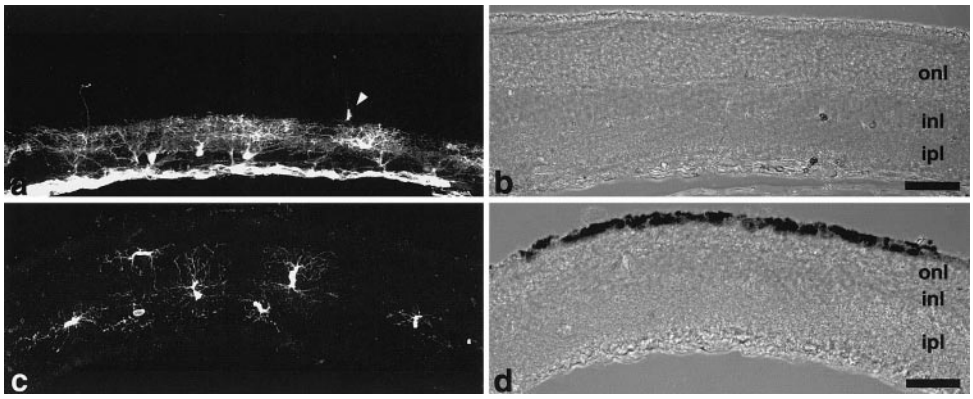

FIGURE 3. The distribution of donor-derived cells in the retinas of adult wild-type mice (**a**) and $\beta2/\beta1$ knock-in mutants (**c**) 1 month after transplantation of spinal precursor cells. In the wild-type (**a**), a layer of EGFP-positive, donor-derived cells was attached to the inner limiting membrane. Several EGFP-positive cells were located in the inner plexiform layer (ipl) and extended a complex network of processes within this layer (**a**). The cell body of one donor cell (*arrowhead* in **a**) was located in the inner nuclear layer (inl). Donor-derived cells in the retina of a $\beta2/\beta1$ knock-in mutant (**c**) also revealed a complex cytoarchitecture and extended numerous processes throughout the host retina. Several cells were located in the inner plexiform layer. EGFP-positive cells in mutant retinas were also located in the inner nuclear layer and at the interface between the inner nuclear layer and outer nuclear layer (onl, **c**). (**b**, **d**) Phase-contrast images of (**a**) and (**c**), respectively. Bar, 50 μm.

tive for EGFP and GFAP) were also detected in the nerve fiber and ganglion cell layers of wild-type and mutant mice (Figs. 5a–c and not shown).

We have previously demonstrated extensive myelination of the normally nonmyelinated intraretinal segments of ganglion cell axons after transplantation of oligodendrocyte progenitor cells and neural precursors into the retina of young rats[29] and mice,[19] respectively. We therefore studied whether grafted precursor cells are also capable of differentiating into myelin-forming oligodendrocytes in adult host retinas. A population of EGFP-positive cells with a characteristic cytoarchitecture was detectable 4 weeks after transplantation in wild-type and mutant retinas. The cell bodies of these cells were usually located in the inner plexiform layer, with processes extending toward the nerve fiber layer (Figs. 6a, 6e). In the nerve fiber layer, processes appeared to terminate in fascicles of ganglion cell axons that were also EGFP immunoreactive (Fig. 6a, 6e). A notable finding was that EGFP positivity in these axon fascicles was invariably colocalized with MAG immunoreactivity (compare Figs. 6a and 6b or 6e and 6f; see Figs. 6c and 6g). Expression of MAG was never observed in EGFP-negative axon fascicles (Fig. 6 and not shown). These observations identify a subpopulation of EGFP-positive cells as myelin-forming oligodendrocytes and suggest that the EGFP fluorescence in the nerve fiber layer is related to donor-derived myelin sheaths that surround ganglion cell axons of the host.

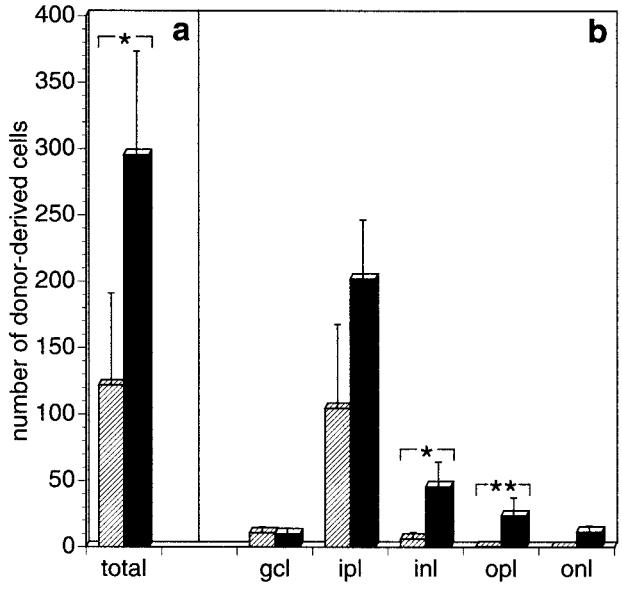

FIGURE 4. The number of donor-derived cells in adult retinas of wild-type and $\beta2/\beta1$ knock-in mice 1 month after transplantation of spinal precursors. Retinas of $\beta2/\beta1$ knock-in mutants (**a**, *black bar*) contained significantly more EGFP-positive cells than retinas of age-matched wild-type mice (**a**, *hatched bar*). Analysis of individual retinal layers (**b**) revealed similar numbers of EGFP-positive cells in the nerve fiber-ganglion cell layer (gcl) and inner plexiform layer (ipl) of both genotypes. In contrast, numbers of donor-derived cells in the inner nuclear layer (inl) and outer plexiform layer (opl) were significantly higher in mutant retinas than in wild-type hosts. In the outer nuclear layer (onl), EGFP-positive cells were found in $\beta2/\beta1$ knock-in mutants, but not in wild-type mice. *Bars* represent the mean value ($\pm$SEM) of six animals. $^*P < 0.05$; $^{**}P < 0.01$, according to the Mann-Whitney test.

Only a few EGFP- and MAG-immunoreactive axon fascicles were detectable in wild-type and $\beta2/\beta1$ knock-in retinas 1 month after transplantation. However, when host retinas were analyzed 6 months after transplantation, colocalization of EGFP and MAG was detectable in large areas of the nerve fiber layer. Host retinas were flatmounted to illustrate this widespread association of EGFP and MAG immunoreactivity with axon fascicles (Fig. 7). In these preparations, faint EGFP positivity was associated over long distances with the majority of axon fascicles (Fig. 7a), and incubation of the tissue with MAG antibodies revealed intense immunoreactivity in all EGFP-positive axon fascicles (compare Figs. 7a and 7b).

Light microscopic inspection of wild-type retinas 6 months after transplantation revealed the presence of myelin sheaths in many axon fascicles of the nerve fiber layer (Fig. 8). Some of these fascicles were heavily myelinated, with the majority of axons being surrounded by myelin sheaths (Fig. 8). Electron microscopic analysis confirmed extensive myelination of the nerve fiber layer and revealed the presence of ultrastructurally intact CNS myelin sheaths (not shown). Together, our data demonstrate differentiation of grafted neural precursors into myelin-forming oligodendrocytes in adult host retinas. Results are also indicative of a progressive, albeit slow, myelination of host axons, ultimately resulting in extensive myelination of the adult nerve fiber layer after extended posttransplantation intervals.

To study differentiation of grafted cells into neurons, we performed double-immunohistochemistry using EGFP antibodies and antibodies to neuron-specific antigens, including neurofilament, β-tubulin type III, and PGP 9.5. None of these neuronal markers was colocalized with EGFP fluorescence. For instance, we screened numerous EGFP-positive cells in $\beta2/\beta1$ knock-in retinas (Fig. 9a) for expression of PGP 9.5 (Fig. 9b). None of the donor-derived cells expressed detectable levels of

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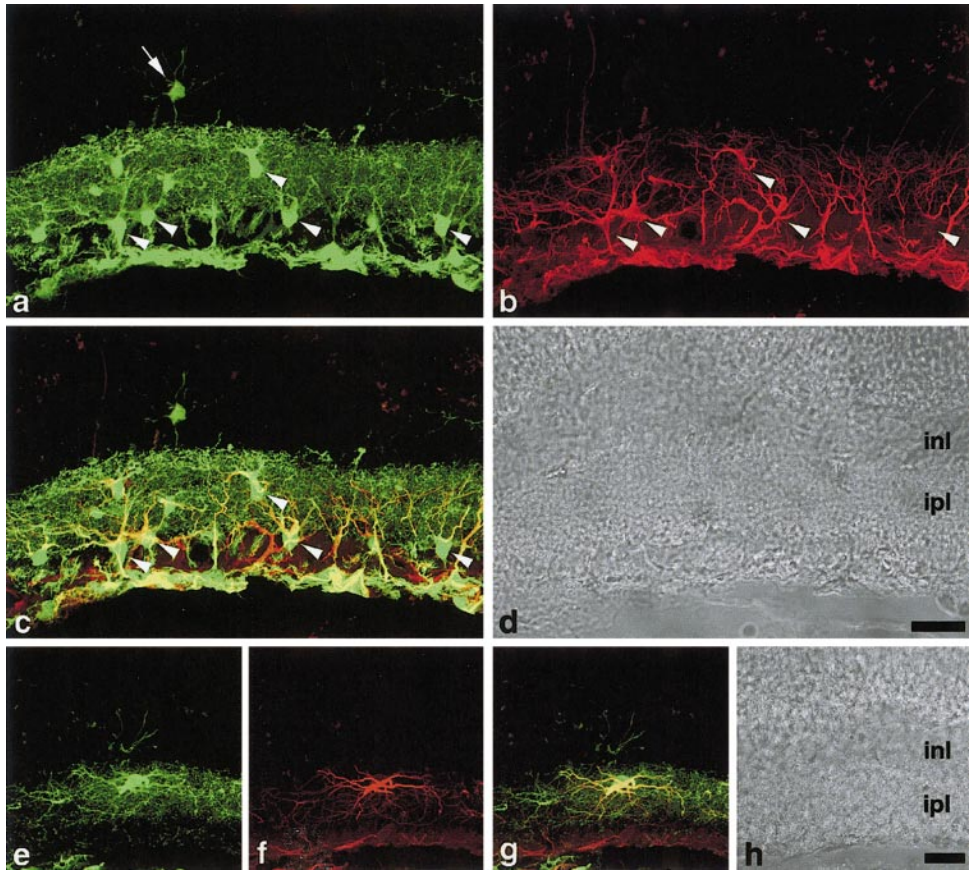

FIGURE 5. Identification of donor-derived astrocytes 1 month after transplantation of spinal precursor cells into the retina of adult wild-type mice. The majority of EGFP-positive, donor-derived cells in a wild-type retina were located in the inner plexiform layer (ipl) and elaborated a dense network of processes (**a**). Immunostaining with GFAP antibodies (**b**) identified several of these donor-derived cells as astrocytes (*arrowheads* in **a**, **b**). Overlay of the EGFP and GFAP images demonstrates colocalization of both proteins in several cells (**c**). An EGFP-positive (**a**, *arrow*) but GFAP-negative (**b**) cell is shown in the inner nuclear layer (inl) of the host retina. An EGFP- (**e**) and GFAP-positive (**f**) astrocyte in the inner plexiform layer of a wild-type mouse. (**g**) Merged image of (**e**) and (**f**). (**d**, **h**) Phase-contrast images of (**a–c**) and (**e–g**), respectively. Bar, 25 μm.

this neuron-specific antigen (compare Figs. 9a and 9b), whereas endogenous horizontal, amacrine, and ganglion cells of the host were intensively PGP 9.5 immunoreactive (Fig. 9b). However, a few donor-derived cells in wild-type and mutant retinas did not correspond to oligodendrocytes, as judged from their cytoarchitecture, and did not express detectable levels of

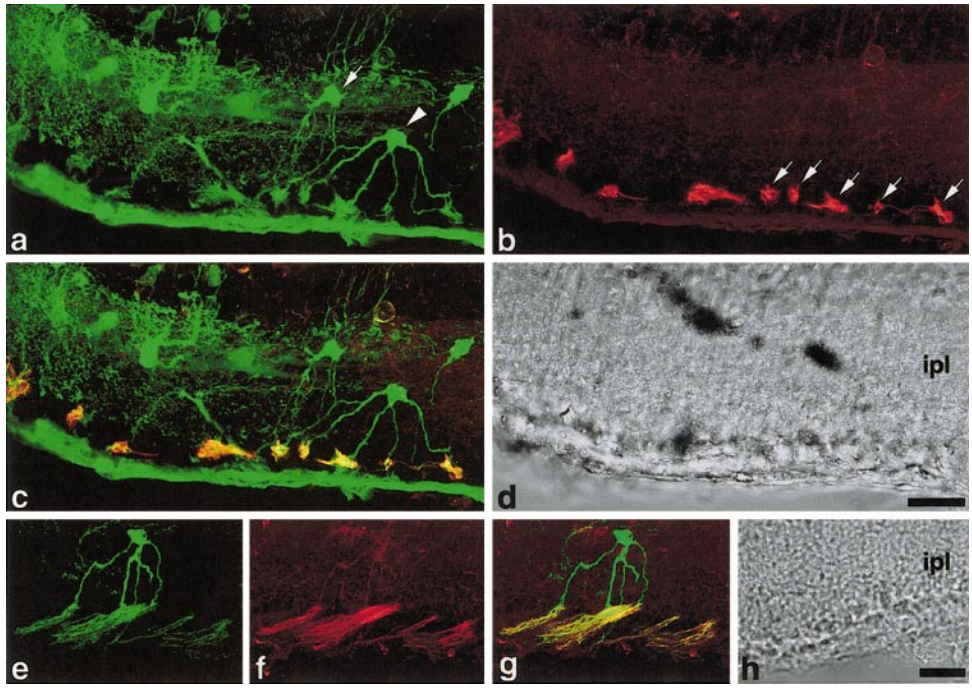

FIGURE 6. Identification of donor-derived oligodendrocytes 1 month after transplantation of spinal precursors into the retina of adult wild-type and $\beta2/\beta1$ knock-in mice. Numerous EGFP-immunoreactive cells were located in the inner plexiform layer (ipl) of a wild-type retina (**a**). Their processes were mainly restricted to this layer, but some donor cells also extended processes into the inner nuclear layer. One EGFP-positive cell (**a**, *arrowhead*), located with its cell body at the upper margin of the inner plexiform layer, extended processes toward the nerve fiber layer. The processes appeared to terminate on fascicles of ganglion cell axons (**a**). A second EGFP-positive cell, located in proximity with the first, also extended processes toward the nerve fiber layer (**a**, *arrow*). Immunostaining of the tissue with MAG antibodies revealed intense immunoreactivity in association with axon fascicles (**b**, *arrows*) in the nerve fiber layer. (**c**) Merged image of (**a**) and (**b**), confirming termination of EGFP-positive processes on MAG-immunoreactive fascicles. EGFP-positive cells (**e**), located in the inner plexiform layer and terminating with their processes on MAG-immunoreactive fascicles in the nerve fiber layer (**f**), were also present in the retina of $\beta2/\beta1$ knock-in mutants. (**g**) Merged image of (**e**) and (**f**). (**d**, **h**) Phase-contrast images of (**a–c**) and (**e–g**), respectively. Bar, 25 μm.

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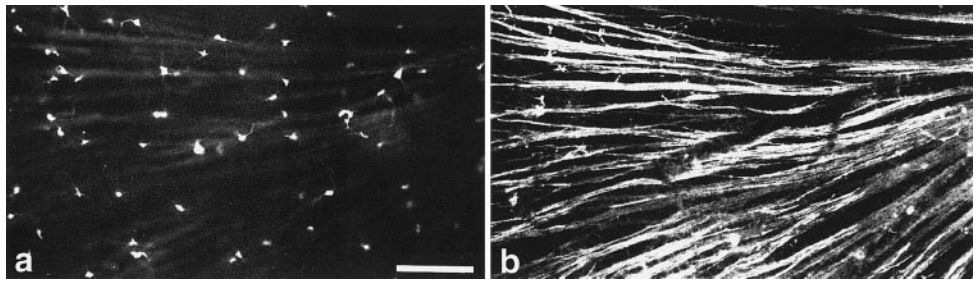

FIGURE 7. Localization of EGFP- and MAG-immunoreactivity in a flatmounted wild-type retina 6 months after transplantation of spinal precursor cells. Numerous EGFP-positive cell bodies were located at the vitread side of the retina, and faint EGFP immunoreactivity was associated with fascicles of ganglion cell axons (**a**). Colocalization of EGFP positivity with intense MAG immunoreactivity in axon fascicles (**b**) indicated myelination of large areas of the host nerve fiber layer by donor-derived oligodendrocytes. Bar, 100 μm.

GFAP. These cells may correspond to immature, undifferentiated cells or to donor-derived nerve cells that escaped identification with the markers used in this study.

DISCUSSION

In the present study, we transplanted neural precursor cells, isolated from the spinal cord of EGFP-transgenic mouse embryos, into normal and dystrophic retinas of adult wild-type and $\beta 2/\beta 1$ knock-in mice, respectively. Grafted cells showed widespread integration into the host tissue, survived for up to 6 months, and differentiated into neural cell types.

Quantitative analysis of recipient tissues revealed a significantly increased number and a more widespread distribution of donor cells in $\beta 2/\beta 1$ knock-in retinas than in wild-type retinas. An obvious assumption is that cellular and molecular differences between wild-type and mutant retinas account for the more pronounced integration of grafted cells into $\beta 2/\beta 1$ knock-in retinas. For instance, it has recently been demonstrated that intravitreally grafted hippocampal precursor cells integrate into the dystrophic retina of adult rats, whereas integration into wild-type retinas requires mechanical lesioning of the retina.[30] It has been suggested that the improved accessibility of the mutant retina may be related to alterations of Müller cells that enable grafted cells to cross the inner limiting membrane and infiltrate the retina.[30] Elevated levels of cell recognition molecules (Bartsch et al., unpublished observations, 2001) or trophic factors[31–33] in pathologically altered retinas may also explain the improved integration and increased numbers of grafted precursors, in that these molecules may stimulate migration and/or proliferation or may support survival of donor cells.

The total number of donor cells in host retinas was low compared with the number of cells used in our transplantation experiments. Because we found no evidence for immunologic rejection or degeneration of grafted cells, we conclude that only a limited number of cells gained access to the host retina at the time of transplantation. In fact, we found numerous EGFP-positive cells in the vitreous of both genotypes, with many donor cells being attached to the inner surface of the recipient retina. It is interesting in the present context that myelination of the nerve fiber layer of recipient retinas was limited 1 month after transplantation but was extensive 6 months after transplantation (see later discussion). This finding also argues against extensive degeneration of donor cells, but instead demonstrates long-term survival of grafted cells in adult host retinas. Moreover, it is indicative of continuous proliferation, migration, and neural differentiation of grafted precursors within the recipient tissue.

Analysis of recipient wild-type and mutant retinas with EGFP and GFAP antibodies 1 month after transplantation revealed that a large fraction of grafted precursor cells had differentiated into astrocytes. Donor-derived astrocytes were located in the nerve fiber and ganglion cell layers, the normal positions of retinal astrocytes.[34] However, they were also detectable in retinal layers not normally populated by retinal astrocytes. Ectopically positioned donor-derived astrocytes were particularly numerous in the inner plexiform layer. In a previous study, we have transplanted spinal and striatal precursors into the retina of young postnatal wild-type mice. Different from adult hosts, donor-derived astrocytes in these young recipients were preferentially positioned in the nerve fiber and ganglion cell layer.[19] Young host retinas thus appear to differ from adult host retinas in expressing cues that instruct differentiation of multipotent cells into appropriate cell types in appropriate locations.

Neural precursors grafted into the retina of adult wild-type and mutant mice also differentiated into oligodendrocytes, a cell type not normally present in the mouse retina. Electron microscopic analysis revealed that donor-derived oligodendrocytes had formed ultrastructurally intact myelin sheaths around ganglion cell axons. These findings confirm the view that the

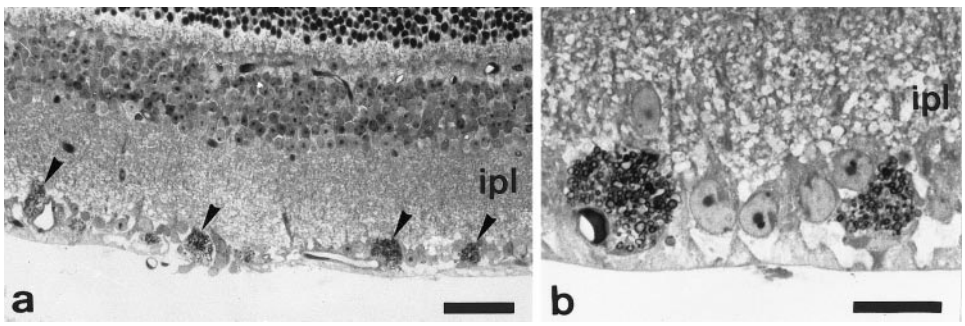

FIGURE 8. Myelinated ganglion cell axons in a wild-type retina 6 months after transplantation of spinal precursors. Several myelinated axon fascicles (**a**, *arrowheads*) were present in the nerve fiber layer (**a**), with a high number of myelin sheaths in each axon fascicle (**b**). Bar, (**a**) 50 μm; (**b**) 25 μm.

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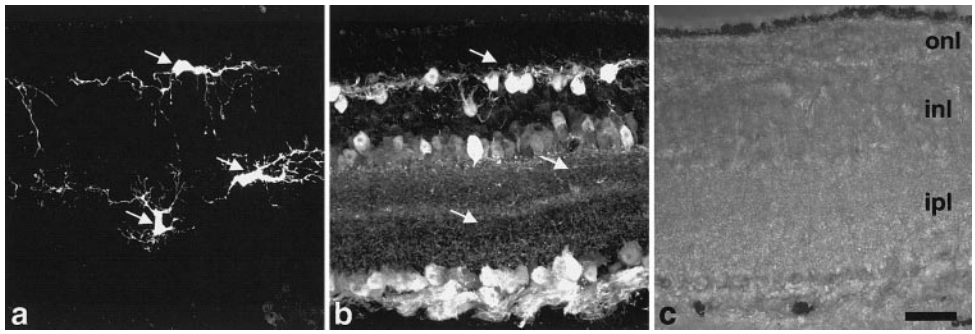

FIGURE 9. Localization of PGP 9.5 immunoreactivity in a $\beta 2/\beta 1$ knock-in retina 1 month after transplantation of spinal precursors. Donor-derived cells were situated in the inner plexiform layer (ipl) and inner margin of the outer nuclear layer (onl, *arrows* in **a**). One of these cells (*uppermost cell* in **a**) extended processes into the outer plexiform layer of the host retina. Expression of PGP 9.5 was restricted to horizontal, amacrine, and ganglion cells in the host tissue (**b**). A comparison of (**a**) and (**b**) demonstrates PGP 9.5 negativity (*arrows* in **b**) of all donor-derived cells. (**c**) Phase contrast image of (**a**) and (**b**). inl, inner nuclear layer. Bar, 25 μm.

normally nonmyelinated intraretinal segments of ganglion cell axons are, in principle, competent to become myelinated once myelin-forming glial cells have access to the retinal nerve fiber layer.[19,29] They thus support the hypothesis that nonneuronal factors yet to be identified at the lamina cribrosa prevent migration of oligodendrocyte progenitor cells from the optic nerve into the retina, and, as a consequence, myelin formation within the retina.[19,29,35–38]

We have recently grafted spinal and striatal precursors into the retina of young postnatal wild-type mice and observed differentiation of a significant fraction of donor cells into oligodendrocytes.[19] One month after transplantation, a considerable portion of the host nerve fiber layer was myelinated by donor-derived oligodendrocytes. After 4 months, virtually the entire nerve fiber layer was myelinated, demonstrating progressive myelination of the host tissue over extended periods.[19] In the current study, we used adult hosts and, compared with young postnatal recipients, observed significantly fewer oligodendrocytes and myelin sheaths 1 month after transplantation of spinal precursor cells. However, large areas of the nerve fiber layer were myelinated 6 months after transplantation, demonstrating progressive and ultimately extensive intraretinal myelination also in adult recipients. It thus appears that the nerve fiber layer of adult hosts becomes as extensively myelinated as the nerve fiber layer of young postnatal hosts, although myelination proceeds more slowly in adult than in young recipients. These observations are in line with a recent study demonstrating that remyelination of experimentally demyelinated axons in the aged adult CNS occurs as extensively as in the young adult CNS, but at a significantly slower rate.[39]

Evidence for differentiation of grafted precursors into nerve cells was not obtained. Double-immunolabeling experiments using antibodies to EGFP and neuronal antigens (i.e., β-tubulin type III, neurofilament, or PGP 9.5), all failed to identify donor-derived neurons in wild-type and mutant retinas. However, a few EGFP-positive donor cells did not express GFAP and did not correspond to oligodendrocytes, as judged from their location and cytoarchitecture. For instance, some EGFP-positive and GFAP-negative cells were located in the outer nuclear layer and extended horizontally oriented processes into the outer plexiform layer. Although the identity of these few cells remains unknown, they may correspond to immature cells that had not yet expressed markers for mature glial cells and/or to donor-derived nerve cells that escaped identification with the neuronal markers used in this study.

Exclusive or preferential differentiation of multipotent precursors into astrocytes or oligodendrocytes may be expected in a non-neurogenic CNS region characterized by a prominent reactive astrogliosis and the presence of nonmyelinated axons. However, fundamentally different results have been obtained with adult hippocampal precursors that were grafted into the dystrophic retina of adult Royal College of Surgeons (RCS) rats[30] or into the mechanically lesioned retina of adult wild-type rats.[40] Exclusive differentiation of hippocampal precursors into nerve cells was observed in the RCS retina,[30] whereas differentiation into neurons and astrocytes was observed in the mechanically lesioned wild-type retina.[40] Evidence for the presence of significant numbers of donor-derived oligodendrocytes was not reported in either of these studies. The reasons for the different fate of embryonic spinal versus adult hippocampal precursors in adult retinas are presently unknown. It is possible that the different fate of spinal and hippocampal precursors is related to the use of different species (mice versus rats) as a host. It is also possible that the different phenotypes of recipient retinas account for the different fate of both precursor populations. However, it should be noted that preferential neuronal differentiation of hippocampal precursors occurs in such diverse recipient tissues as the dystrophic retina of RCS rats and the mechanically lesioned retina of wild-type rats. Furthermore, exclusive differentiation of spinal precursors into glial cells and preferential differentiation of hippocampal precursors into nerve cells was observed, not only in mutant mice and rats, but also in wild-type mice (the present study) and rats,[40] respectively. It is therefore tempting to speculate that differences between the two precursor populations, either intrinsic or related to different culture conditions, account for the divergent fate of spinal and hippocampal precursors. For example, hippocampal precursors were expanded using FGF-2,[30,40] whereas spinal precursors were expanded in the presence of FGF-2 and EGF (the present study). There is evidence for the presence of distinct EGF- and FGF-2-dependent neural precursor populations. Furthermore, it has been demonstrated that EGF, compared with FGF-2, favors glial differentiation of neural precursors, both in vitro and in vivo.[41–46]

The present and other studies[30,40] demonstrate robust integration, long-term survival, and neural differentiation of heterotopically transplanted neural precursor cells in the normal and pathologically altered adult retina. However, differentiation of heterotopically grafted precursors into retina-specific cell

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types was not observed in any of these studies. The recent isolation, cultivation, and characterization of retinal precursor cells from the adult eye of various mammalian species, including humans, is therefore of particular interest.[47] Retinal precursor cells are pigmented cells located in low numbers at the ciliary margin. They display the two cardinal features of stem cells: multipotentiality and the capacity for self-renewal. When retinal precursor cells are induced to differentiate in vitro, they give rise to cells expressing antigens characteristic of Müller cells, bipolar neurons, and rod photoreceptor cells.[47] Retinal precursor cells have also been isolated from adult rats.[48] It is now of obvious interest to obtain information about the fate of grafted retinal precursor cells in the normal and dystrophic adult retina.

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