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One strategy to restore vision in retinitis pigmentosa and age-related macular degeneration is cell replacement. Typically, patients lose vision when the outer retinal photoreceptor layer is lost, and so the therapeutic goal would be to restore vision at this stage of disease. It is not currently known if a degenerate retina lacking the outer nuclear layer of photoreceptor cells would allow the survival, maturation, and reconnection of replacement photoreceptors, as prior studies used hosts with a preexisting outer nuclear layer at the time of treatment. Here, using a murine model of severe human retinitis pigmentosa at a stage when no host rod cells remain, we show that transplanted rod precursors can reform an anatomically distinct and appropriately polarized outer nuclear layer. A trilaminar organization was returned to *rd1* hosts that had only two retinal layers before treatment. The newly introduced precursors were able to resume their developmental program in the degenerate host niche to become mature rods with light-sensitive outer segments, reconnecting with host neurons downstream. Visual function, assayed in the same animals before and after transplantation, was restored in animals with zero rod function at baseline. These observations suggest that a cell therapy approach may reconstitute a light-sensitive cell layer de novo and hence repair a structurally damaged visual circuit. Rather than placing discrete photoreceptors among preexisting host outer retinal cells, total photoreceptor layer reconstruction may provide a clinically relevant model to investigate cell-based strategies for retinal repair.

Degeneration of the outer retina resulting in sight loss may occur in retinitis pigmentosa (RP) and other retinal degenerations in which there is a progressive loss of photoreceptor cells from the outer nuclear layer (ONL). A potential treatment for patients who are blind from outer retinal degeneration would be photoreceptor transplantation using a cell therapy approach. The observation that previously blind patients could read after stimulation by subretinal electrodes (1) indicates that the downstream circuitry of the human inner retina retains a capacity to be reactivated to restore vision long after all photoreceptors are lost.

transplantation, with the formation of light-sensitive outer segments (OS) containing enzymes critical for light sensitivity. Furthermore, the cells would need to reconnect synaptically with residual downstream retinal neurons for light-evoked signals to be relayed to central targets.

Photoreceptor replacement in the absence of the host ONL remains relatively uninvestigated, and it is not known if precursor cell transplantation could be a successful approach for the end-stage degenerate host with a zero anatomical and functional baseline. Rather than adding more photoreceptor cells into a preexisting host ONL, we aimed instead to investigate whether a functional ONL could be reconstructed *de novo* by precursor cell transplantation.

To model the clinical scenario of complete rod photoreceptor degeneration, we used C3H/HeNHsd (*rd1*) host mice aged 10–12 wk, which were homozygous for the retinal degeneration allele of the rod-specific cGMP phosphodiesterase $\beta 6$ subunit (*Pde6b^{rd1}*) (4). In this fast-degenerating model of severe human RP, the death of all rods occurs by 3 wk of age, and beyond this time almost all of the ONL is lost. We used Tg(*Nrl-L-EGFP*) mice (5) as donors, in which green fluorescent protein (GFP) expression is restricted to postmitotic rod precursors and mature rods as a consequence of the *Nrl* promoter, which would therefore allow us to track donor rods after transplantation into the host. Precursors were harvested from postnatal day (P) 3–4 mice, coincident with the period of rod genesis and *Nrl* expression, as cells at this stage have been shown to survive transplantation (3, 6). We optimized the transplantation technique for the degenerate retina lacking the ONL and found that standardized cell transplantation could be consistently achieved in the fragile and almost transparent host retina (Fig. S1). Two weeks after transplantation, we found numerous GFP-positive donor cells appropriately interposed between the host inner nuclear layer (INL) and retinal pigment epithelium (RPE), recreating a new ONL up to 10 cells thick in places (Fig. 1*A–F*). On average, $7.9 \pm 6.5\%$ ($n = 8$) of donor cells survived. In contrast to previous reports in which the host ONL was relatively intact (3, 7–11), we

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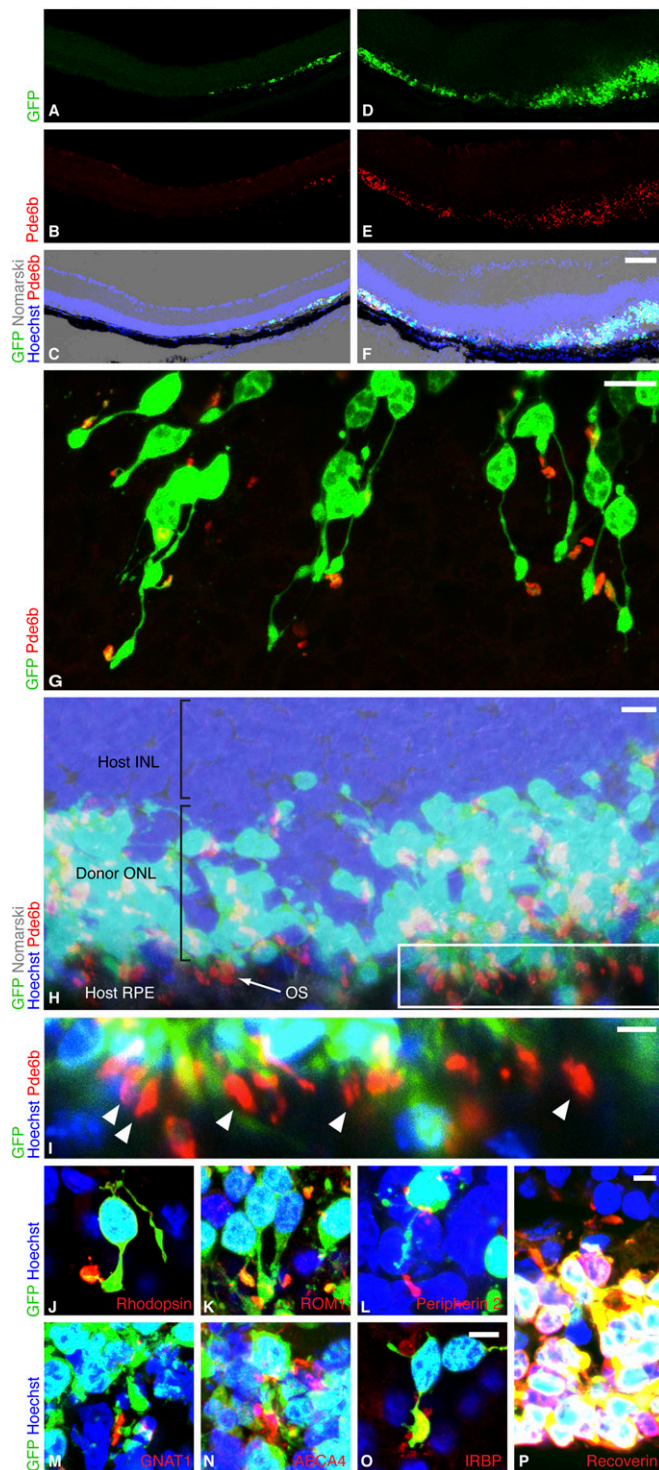


Fig. 1. Rod precursors transplanted into the degenerate host regenerate a new photoreceptor layer. (A–F) Two regions of *rd1* host retina after subretinal transplantation of P3 Tg(*Nrl-L-EGFP*) rod precursors. By 2 wk, the retina had reattached and a new donor-derived ONL, marked by GFP, was formed. A–C show an area where one to three ONL rows were formed, and D–F show up to 10 rows. An internal nontransplant control region lacking an ONL is shown in the left half of A–C. (Scale bar, 75 μ m.) (G) As a sign of maturation, donor-derived GFP-positive rods formed slender processes extending from the cell body and expressed phosphodiesterase $\beta 6$ (Pde6b) sequestered in discrete OS. (Scale bar, 10 μ m.) (H) In many regions, Pde6b-positive OS were appropriately polarized with respect to host RPE, which is the optimal configuration for rod OS maintenance. (Scale bar, 10 μ m.) Boxed region of H enlarged in I shows OS (arrowheads) aligned in rows against host

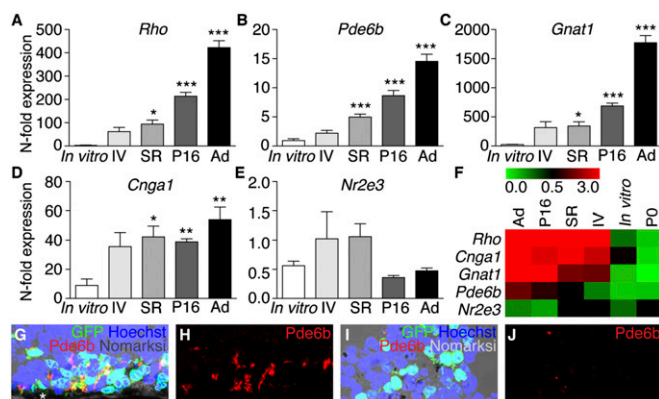
observed no significant migration of cells into the residual nuclear layers of the host. Furthermore, none of the transplanted rods that we observed adopted the normal photoreceptor profiles that have been reported in other studies where exogenous fluorescent precursors coexisted with numerous host ONL photoreceptors at the time of transplantation (3, 7–11). In a longitudinal *in vivo* analysis of 12 eyes, 33.3% had surviving donor cells at week 12, the last time point assessed (Fig. S1 E–L).

We asked if precursors relocated to the subretinal space of the severely degenerate retina could follow normal development and elaborate OS, thereby completing the final steps of differentiation into mature light-sensing rods. The OS is the photosensitive part of the photoreceptor and normally houses enzymes required for phototransduction and the visual cycle. In transplanted donor cells, we found widespread immunoreactivity against Pde6b, a phototransduction enzyme normally localized in the OS, which was absent in the *rd1* hosts and was therefore reliably donor-cell-derived. Pde6b was consistently sequestered in a discrete OS (Fig. 1G and Table S1) that was, in many regions, elongated and appropriately orientated toward RPE (Fig. 1H–I). This configuration is known to support the physiological function of photoreceptors as they are dependent on RPE cells. The presence of appropriately sequestered Pde6b indicated directional protein transport in the connecting cilium (12) and constituted evidence of donor rod maturation. OS were formed in $76.8 \pm 18.9\%$ (mean \pm SD) of surviving donor rods (i.e., from about 6% of transplanted cells). OS were significantly more often positioned in the outer part of the donor-derived ONL against the host RPE than in the inner ONL against the host INL ($63.0 \pm 6.1\%$ vs. $25.5 \pm 3.5\%$, mean proportion \pm SEM, $t_{10} = 4.30$, $P = 0.002$, $n = 11$ discrete ONL regions in four experiments). This preferential orientation suggested an interaction between these cell layers. As further evidence of maturation, donor cells expressed mature rod- and photoreceptor-specific markers (Fig. 1J–P) 2 wk after transplantation.

To investigate whether the degenerate subretinal niche was a permissive environment for the completion of rod precursor development, we quantified rod-specific gene expression by quantitative RT-PCR (qRT-PCR) mRNA analysis from eyes containing P2–3 precursors transplanted *in vivo* compared with cells maintained in culture for 2 wk. Significantly, we observed that precursors transplanted into degenerate hosts resumed developmental expression of rod-specific genes required for OS function (*Pde6b*, *Rho*, *Gnat1*, *Cnga1*) (Fig. 2A–F and Table S2). This was not observed in precursors maintained *in vitro*, suggesting that the degenerate subretinal niche was able to influence OS-related gene expression beyond that which is pre-programmed at the time of harvest. This correlated with the identification of OS histologically in transplanted precursors (Fig. 2G–J). Also, the expression of *Rho*, *Cnga1*, and *Gnat1* was restored in *rd1* eyes after retinal reconstruction (Fig. S2). Together, these data show that donor-derived rod cells had the capability to differentiate and form OS after being relocated from their native environment to the end-stage degenerate subretinal space.

To transmit visual signals, transplanted cells would need to connect with the residual neuronal circuitry in the host. To explore donor–host connections, we used fluorescent red Tg(CAG-*DrRed**MST1)*Nagy/J* murine hosts (13) aged 10–12 wk that were on an *rd1* background. Thus, these fast-retinal-degeneration

RPE. (Scale bar, 5 μ m.) (J–N) As further evidence of maturation, rhodopsin, rod outer segment membrane protein 1 (ROM1), peripherin 2, guanine nucleotide-binding protein G(t) subunit $\alpha 1$ (GNAT1), and ATP-binding cassette subfamily A member 4 (ABCA4) were localized in outer segments. (O) Donor cells expressed interphotoreceptor retinoid-binding protein (IRBP), a secreted protein critical for ONL maintenance, and (P) were positive for the photoreceptor marker recoverin. (Scale bar, 5 μ m for J–P.)



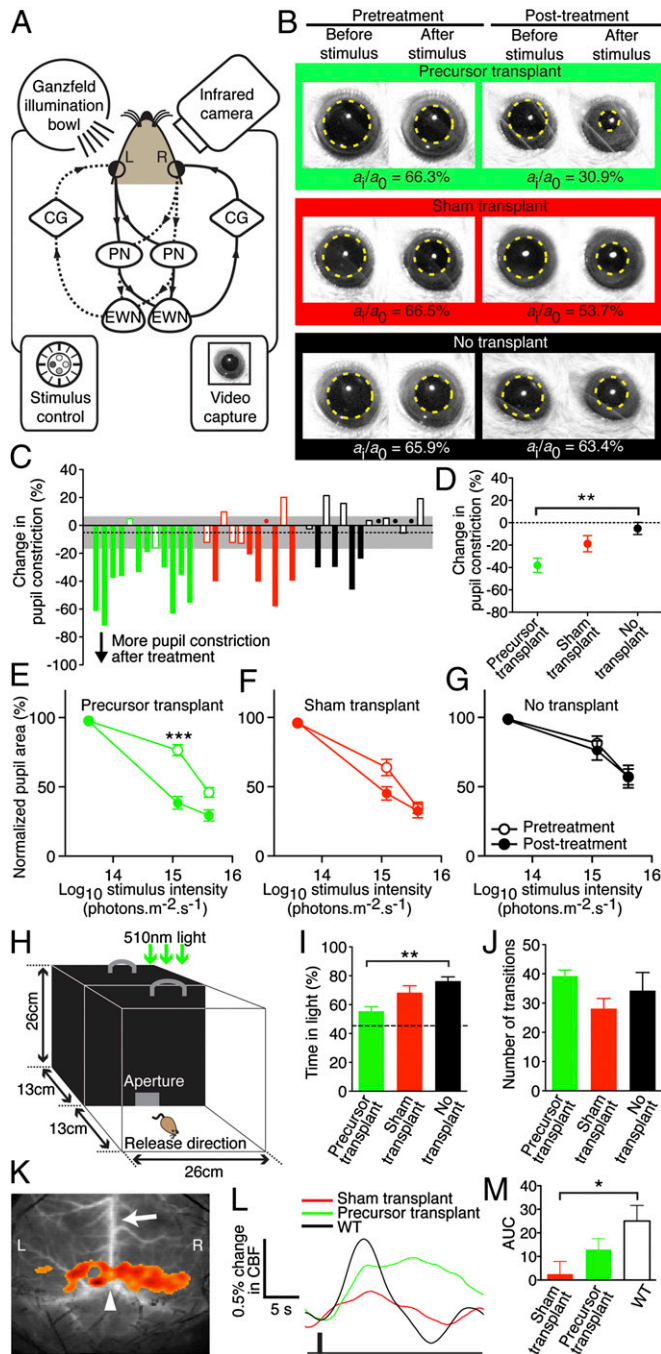


Fig. 4. Rod precursor transplantation restores visual function to eyes with complete rod degeneration. (A) Retinal illumination results in signal transmission to both pretectal nuclei (PN) and Edinger–Westphal nuclei (EWN). Impulses are relayed via the ciliary ganglia (CG) and both pupils constrict. Solid and dashed lines show reflex arcs triggered by left (L) and right (R) eye stimulation, respectively. (B) Representative pupil images. (C) Overview of the change in pupil constriction from before to after treatment in all mice at 1.21×10^{15} photons·cm⁻²·s⁻¹. Green, red, and black denote precursor, sham, and no transplant controls, respectively. Dots indicate values close to zero and dashed line indicates the mean change with 95% confidence interval (gray) of the non-transplanted controls. The PLR was measured in the same eye 3 d before and 2 wk after subretinal transplantation of 3×10^5 cells. PLR was expressed as normalized pupil area as previously described (3). (D) Precursor transplantation improved pupil constriction, compared with no transplant controls ($F_{2,34} = 7.264$, $P = 0.002$, $n = 11$ –14 each; ** $P = 0.001$ post hoc). (E) Paired analysis before versus after treatment showed that at the median light stimulus intensity, the PLR improved significantly in precursor-treated animals (**** $t_{11} = 5.95$, $P < 0.0001$, $n = 12$). (F and G) The PLR did not

activity toward the mean wild-type (WT) level (Fig. 4 *I–J* and Movies S1 and S2). Furthermore, laser speckle imaging (24) showed blood flow changes in the visual cortices of precursor-transplanted *rd1* mice that were not seen in sham-treated controls (Fig. 4 *K–M*). In all, these data are consistent with a significant recovery of visual function by photoreceptor layer reconstruction after host rod loss.

Previous reports examining rod precursor integration into a preexisting ONL showed peak efficacy with early postnatal cells (3, 25). Conversely, a recent study suggested that adult donor rods could be transplanted (6), and adult rods have been shown to exert a trophic effect on host cone cells (26). Hence we examined functional effects in degenerate retinæ using older donors. Adult (P30–P60) and late-developing (P14) donor cells survived after subretinal transplantation, but did not form a functional ONL (Fig. S6). We compared host cone morphology and opsin levels between P3 precursor-treated and sham-treated *rd1* mice, but no differences were observed at 2 wk ($t_{10} = 0.50$, $P = 0.6$, $n = 6$ in each group) (Fig. S7). Collectively, these observations are in keeping with a visual improvement arising directly from transplanted rod precursors, rather than indirectly through host cone rescue.

Discussion

The data show that a functional photoreceptor layer of rods may be recreated de novo by the transplantation of rod precursor cells. In advanced disease typical of the human situation featuring a degenerated host ONL, transplanted cells were able to re-form an outer retinal layer in the correct anatomical location. The cells resumed their developmental program to polarize appropriately and form mature light-sensing outer segments, and synaptic integration at the donor–host interface allowed the transmission of light-evoked responses to the brain.

The development of a discrete OS with sequestered phototransduction enzymes represents a final stage of rod differentiation that has yet to be observed consistently in ES or induced pluripotent stem cell derivatives transplanted into other models or in ES cell-derived optic cups in culture (2, 7, 27–29) (Fig. S8A). We have shown that cells at the *Nrl*-expressing stage have the capacity to resume normal OS development when relocated to the terminally degenerate niche, and so the data reflect the replacement cell therapy paradigm in introducing new light-sensing components into the retina. The formation of elongated and enzyme-localizing outer segments, in many cases apposed to RPE (Fig. S8 B–D), supports the hypothesis that the improvement in visual function is due to the physiological function of repopulated photoreceptors.

The potential for photoreceptor replacement to be translated into humans with complete outer retinal degeneration has not been previously addressed. We used a severe and rapidly degenerating murine model, the *rd1* mouse, in order to effectively model the anatomy in end-stage human retinal degenerations. Models used in previous studies included wild-type (3, 8, 9, 25, 27, 30–33), *rd9*

improve after sham transplantation ($n = 11$) or in untreated controls ($n = 14$) at any stimulus intensity. There were no significant differences between groups at baseline ($P = 0.12$). (*H*) Light-mediated behavior was measured under dim 510-nm illumination ($150 \text{ nW}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$). (*I*) Precursor-transplanted mice spent less time in the lit compartment than *rd1* controls ($F_{2,20} = 7.608$, $P = 0.003$, $n = 7-8$ each; $**P = 0.003$ post hoc). Dashed line indicates mean WT level. (*J*) Anxiety-related behavior (transitions between compartments) was similar across groups. (*K*) Cortical activation map in WT showing increased cortical blood flow (CBF) over L and R visual cortices; arrow, superior sagittal sinus; arrowhead, lambda. (*L*) Averaged L and R CBF time series from all mice after a 4-Hz (1-s) stimulus (black bar). (*M*) Precursor transplantation led to a higher CBF change than sham treatment compared with WT ($F_{2,16} = 4.108$, $P = 0.04$, $n = 5-7$ each; $*P = 0.01$). AUC, area under curve (0-7 s). Error bars, SEM.

(10), *Rho*^{-/-} (3, 28, 33), *Crx*^{-/-} (7), *Crb1*^{rd8/rd8} (8, 31, 33), *Prph2*^{rd2/rd2} (31), *Gucy2e*^{-/-} (8), and *Gnat1*^{-/-} (11) mice. These hosts featured the relative anatomical integrity of the native ONL at the time of precursor transplantation, compared with the end-stage ONL loss in the *rd1* mouse. In this regard, the *rd1* host used in this study was an appropriate model to study the typical situation in advanced human RP, where the need for cell replacement arises when visual symptoms develop due to near-complete outer retinal cell loss. Outer neural retinal replacement would also likely be required as one component of a cell replacement approach for the treatment of advanced age-related macular degeneration. Our data indicate that donor cell survival, maturation, and integration are possible despite the reorganization that is known to occur in advanced degeneration (17).

A critical issue relating to stem cell treatments is whether the restorative effects are mediated by differentiation of the immature cells into a specific cell type or, alternatively, from donor–host cell fusion. Stem and progenitor cells that generate neurons have been shown to generate fused cells (34). In theory, the presence of preexisting host rods at the time of stem or precursor cell transplantation may allow for the fusion of fluorescence-labeled donor cells with host cells and give rise to the appearance of integrated and well-formed donor-derived cells residing among host cells (11). Our experimental design using rodless hosts excluded the possibility of immature donor rod precursors fusing with host rods. The morphological results presented here, including the misaligned OS (Fig. 1*G*) and the absence of long inner fiber formation (Fig. 3*A–H*), may reflect the outcomes to be expected following cell transfer in the absence of coexisting host ONL cells.

The use of the consensual PLR provided evidence not only that the reformed ONL was capable of detecting light, but also that signals were being transmitted to central nervous system targets. There is a complementary contribution from intrinsically photosensitive retinal ganglion cells to the PLR, especially at high irradiances with long stimuli (35). It was important in this study to assess the functional contribution of the newly introduced rods in the treatment group. Consequently, we used a short (0.1 ms) stimulus and measured the PLR at a relatively

short time after stimulus offset (*SI Materials and Methods*) to better isolate the high-amplification rod photoreceptor contribution to the measured PLR. This approach has been validated as a measure of photoreceptor function in studies of human and mouse visual dysfunction due to *Rpe65* mutations (36). The temporal characteristic of the cortical hemodynamic response after precursor transplantation deviates from the WT response; this may be due to alterations in neurovascular coupling mechanisms following reduced neuronal activity associated with loss of visual input before ONL reconstruction.

In summary, transplanted rod precursors may mature and re-create a new functional photoreceptor layer in the degenerate outer retina. These data support the potential use of photoreceptor precursors for the treatment of human blindness due to advanced RP and related conditions.

Materials and Methods

Animals were maintained in the animal facility at the University of Oxford. All experiments were conducted according to the UK Home Office guidelines on the Animal (Scientific Procedures) Act of 1986 and were approved by the University of Oxford Animal Ethics Committee. Donor cells were harvested from P3–P4, P14, P30, or P60 Tg(*Nrl-L-EGFP*) mice (kind gift of Anand Swaroop, National Eye Institute, Bethesda, MD). For sham transplants, retinal cells were harvested from Tg(CAG-DsRed**MST1*)Nagy/J mice (Jackson Laboratories) older than 6 wk, using the Papain Dissociation System (Worthington Biochemical) according to the manufacturer's instructions. Cells were diluted to a final concentration of 2×10^5 cells/μL DMEM for transplantation. Approximately 3×10^5 cells were transplanted in every case. Further experimental details are described in *SI Materials and Methods*.

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