Donor and host photoreceptors engage in material transfer following transplantation of post-mitotic photoreceptor precursors

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Photoreceptor replacement by transplantation is proposed as a treatment for blindness. Transplantation of healthy photoreceptor precursor cells into diseased murine eyes leads to the presence of functional photoreceptors within host retinas that express an array of donor-specific proteins. The resulting improvement in visual function was understood to be due to donor cells integrating within host retinas. Here, however, we show that while integration occurs the majority of donor-reporter-labelled cells in the host arises as a result of material transfer between donor and host photoreceptors. Material transfer does not involve permanent donor–host nuclear or cell–cell fusion, or the uptake of free protein or nucleic acid from the extracellular environment. Instead, RNA and/or protein are exchanged between donor and host cells in vivo. These data require a re-evaluation of the mechanisms underlying rescue by photoreceptor transplantation and raise the possibility of material transfer as a strategy for the treatment of retinal disorders.
Transplantation of healthy donor cells into diseased environments is a promising therapeutic strategy for a wide range of diseases. In the eye, despite different underlying causes, many degenerative disorders lead to the loss of the light-sensitive photoreceptors and blindness. At present, few clinically available treatments are capable of reversing this. Clinical trials for gene-supplementation therapy have shown promise for patients with known genetic defects, but photoreceptor replacement by transplantation is proposed as a broad treatment strategy applicable to many forms of retinal degeneration. Photoreceptor replacement could be useful during the degenerative process, when some photoreceptors remain, for diseases with an unknown aetiology. Alternatively, cell therapy may be applied as a treatment for end-stage disease, when little, if any, of the outer nuclear layer (ONL) of the host retina remains.

We, and others, have shown previously that the transplantation of stage-specific post-mitotic rod photoreceptor precursors carrying a transgenic green fluorescent protein (GFP) label results in the presence of GFP-positive (GFP⁺) rod-like cells within the ONL where photoreceptors normally reside, in wild-type and degenerating murine host retinae. These cells bear the morphological characteristics of mature photoreceptors, including synapse-like structures and outer segments. Importantly, when transplanted into different models of retinal degeneration, GFP⁺ cells within the host ONL demonstrated robust levels of those proteins that were genetically absent in the host photoreceptors. Moreover, single-cell recordings showed these GFP⁺ cells to be light-responsive in a manner very similar to that of normal wild-type photoreceptors. The presence of these cells also correlated with visually evoked activity in the visual cortex and behaviour, when present in sufficiently large numbers.

Strikingly, the developmental stage of the donor cell at the time of transplantation is important; transplantation of embryonic or adult photoreceptors led to poor integration of cells within the host ONL, a finding that holds true for both postnatally derived donor cells and photoreceptor precursors derived from three-dimensional (3D) differentiation of embryonic stem (ES) cells. Altogether, these data provided evidence of robust rescue of photoreceptor function following the transplantation of healthy photoreceptors.

Functional rescue of different tissues in a variety of disease models by stem cells has been widely reported. However, what was previously thought to be stem cell integration and differentiation has since been shown to be cell–cell fusion between the donor stem cell and the host cell in some studies. Classic cell fusion involves the combining of two cells and their nuclei. The two nuclei may remain as separate entities or may slowly fuse to form a single nucleus. The process of nuclear fusion between mature cells is typically slow and takes many days to weeks. In some cases, a third cell is involved; for example, transplantation of neural stem cells into the rodent cortex results in their fusion with mature pyramidal neurons, in a two-step process that appears to be mediated by microglia. Such reports are not uncommon where the donor cell is a stem cell. There are very few examples, however, where fusion occurs between donor and host neurons when both are post-mitotic.

Previously, we, and others, addressed the possibility of cell fusion after transplantation of post-mitotic photoreceptors into the adult host retina using two techniques. In the first, a permanent nuclear label, BrdU, was introduced into a proportion of donor cells before transplantation. We found examples of GFP⁺ photoreceptors within the host retina that bore a single BrdU⁺ nucleus, providing strong evidence that these were donor cells that had migrated into the host retina. Other apparently integrated cells were BrdU⁻ but as only a small proportion of the starting donor cell population carried the label, this was not unexpected. In the second, GFP⁺ donors were transplanted into CFP (cyan fluorescent protein)-transgenic recipient mice. Confocal imaging found GFP⁺ cells within the host retina with GFP⁺/CFP⁻ inner segments. Ader and colleagues reported similar results by using a viral vector to label their donor population with tdTomato and transplanting into eGFP-transgenic recipients. Others have heralded this dual-fluorescent donor/recipient technique as a gold standard for assessing cell fusion. Moreover, analysis of transplanted retinae at different stages post transplantation, ranging from 48 h to 6 weeks, revealed the presence of stereotyped morphologies of donor cells approaching and extending processes to the host retina, before moving across the outer limiting membrane (OLM), a physical barrier whose disruption can lead to significantly increased numbers of donor–reporter-labelled cells within the host retina. Altogether, these findings have been regarded by the oculomotor field as strong evidence supporting the hypothesis that donor cells migrate into the recipient retina and mature and differentiate in situ.

As we have continued to research photoreceptor transplantation in increasing detail, we have made observations that suggest that donor cell migration and integration is not the only mechanism involved. Below, we present data using a wide variety of techniques that indicate that post-mitotic donor and host photoreceptors can engage in the transfer of either RNA and/or protein, resulting in the robust presence of a wide variety of donor-specific proteins in host cells. While the precise transfer mechanism remains unclear, it does not involve classic cell–cell nuclear fusion.

**Results**

**Real-time imaging of integration events.** FAC-sorted GFP⁺ photoreceptor precursor cells from postnatal day 8 (P8) NrGFP B6.Cg-Tg(Nrl-EgFP)1Asw/J (NrGFP) mice were transplanted into adult Prph2rd2/rd2 (ref. 31) hosts. Explanted host retinae were labelled with Mitotracker Orange CMTMRos to visualize the host retinal structure and associated donor cell mass and imaged 72 h post transplantation using 2-photon real-time imaging. Some donor cells appear to move into the host retinae over a period of several hours (Fig. 1; Supplementary Movie 1). Typically, donor cells initially locate to the interphotoreceptor matrix and appear to extend a process toward the OLM, before moving into the host ONL. Movement into the host retina was restricted to the first 1–2 photoreceptor rows and deeper penetration was not observed, although it is possible that such migration occurs over a longer time period than was possible to image here. These data support the occurrence of donor cell migration into the host retina, very similar to that reported for fixed tissue time series.

**Exchange of reporters between donor and host photoreceptors.** In a complementary series of experiments aiming to evaluate donor–host cell interactions, we repeated the fluorescent reporter transplants that we, and others, reported previously, but this time using two different fluorescent labels and analysis by confocal microscopy and flow cytometry. NrGFP donors were transplanted into adult B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J(DsRed) transgenic hosts, which ubiquitously express the fluorescent reporter, DsRed. At 5–6 weeks post transplantation, retinal sections were imaged using confocal microscopy and assessed for localization of GFP⁺ cells within the DsRed host ONL (Fig. 2). Of 157 GFP⁺ cells (N = 5 retinae) examined, 36% of inner segments were identified by two assessors as showing an increase
GFP fluorescence gradually reduced over the imaging period, Mitotracker Orange CMTMRos labelling, which is present in both donor and host cells, views of time frames shown in (b). A dashed line (white) was inserted at the presumptive level of the OLM. (b). Time course of rod precursor cell movement from the IPM into the ONL; penetration through the OLM (white dashed line) is indicated by white arrowheads (see also Supplementary Movie 1). (c,d), High magnification views of time frames shown in (b) depicting migration of the right (c) and left (d) rod precursor cell into the ONL at selected time points. Note that while GFP fluorescence gradually reduced over the imaging period, Mitotracker Orange CMTMRos labelling, which is present in both donor and host cells, persisted in its absence. Scale bars, 10 μm.

Figure 1 | Real-time imaging of transplanted donor precursor cells migrating into host retinae. NrlGFP post-mitotic photoreceptor precursor donor cells (green) were transplanted into 12 weeks old Prph2Δ22/Δ22 mice and explanted retinae were examined by real-time 2-photon fluorescence live imaging 3 days post transplantation (retinae, including donor and host cells, were acutely labelled with Mitotracker Orange CMTMRos (red) before the recording). (a), At the start of the time-lapse recordings, sub-retinally transplanted rod precursor cells were frequently observed at the level of the IPM or apical to the retinal tissue. A dashed line (white) was inserted at the presumptive level of the OLM. (b), Time course of rod precursor cell movement from the IPM into the ONL; penetration through the OLM (white dashed line) is indicated by white arrowheads (see also Supplementary Movie 1). (c,d), High magnification views of time frames shown in (b) depicting migration of the right (c) and left (d) rod precursor cell into the ONL at selected time points. Note that while GFP fluorescence gradually reduced over the imaging period, Mitotracker Orange CMTMRos labelling, which is present in both donor and host cells, persisted in its absence. Scale bars, 10 μm.

in GFP fluorescence and a concomitant decrease in DsRed fluorescence (GFP+/DsRed−; Fig. 2a,d), indicative of integrated cells. However, in 60% of cells the DsRed signal was either unchanged or increased relative to neighbouring cells (GFP+/DsRed+; Fig. 2a,e). Although stringent confocal settings were used (see the ‘Methods’ section), the dense packing of rod photoreceptors means that there is very little cytoplasm, making assessments of co-localization of cytoplasmic labels prone to error, particularly overestimation of co-localization.

Nevertheless, when examining cell bodies of the same population, 21% were GFP+/DsRed− (Fig. 2b,f), leaving 76% of GFP+ cells being additionally DsRed+ (Fig. 2b,g). The remaining cells (<4%) represent those for which there was no consensus between assessors. Interestingly, when the GFP+/DsRed− cells (integrated) were assessed with respect to their position in the host ONL, there was significant trend for them to be located within the first two photoreceptor rows, closest to the OLM (Fig. 2h,i). In addition, we examined those donor cells that failed to integrate but remained in the subretinal space (SRS). Most expressed GFP alone but a small number also expressed DsRed, albeit at low levels (Fig. 2j). These observations suggest that while some donor photoreceptors integrate into the host ONL, donor-reporter RNA and/or proteins might also be able to move not only from donor to host photoreceptors but also vice versa.

The difference in the proportion of GFP+/DsRed+ cell bodies, compared with inner segments, within the same population of cells demonstrates the limitations of this method of analysis. We repeated the experiment, this time dissociating the isolated host neural retinae and assessing the resulting cell populations using flow cytometry (Fig. 3 and Supplementary Fig. 1). The gates were set on the basis of wildtype, DsRed and NrlGFP controls (Fig. 3b–d). Of 18 host retinae examined, the total number of GFP+ cells collected per host eye ranged between 120 and 10,575 cells (mean = 2,130 ± 2,772 cells; Fig. 3a). Of these, 18.7% (±24.9; median value = 4.7%) were GFP+/DsRed−, however 81.4% (±24.8; median value = 95.3%) of GFP+ cells were also DsRed+. GFP+/DsRed− cells had slightly higher levels of GFP when compared with GFP+/DsRed+ cells, as demonstrated by mean fluorescence intensity (Fig. 3e,f and Supplementary Fig. 1). Taken together with the confocal data, the GFP+/DsRed− population likely corresponds to integrated cells, although a small proportion may reflect donor cells located in the SRS that had adhered to the neural retina. We excluded the possibility that GFP+/DsRed− cells included resident or infiltrating macrophages that had phagocytosed GFP, by using CD45 staining. Less than 0.016% of GFP+/DsRed+ cells co-stained with CD45 in any given sample (N = 8 retinal samples; Fig. 3g), confirming that they were not macrophages and/or other white blood cells.

These analyses confirm the presence of GFP+/DsRed− cells within the host ONL that are most likely donor cells that migrate into the recipient retina. However, the high proportion of GFP+/DsRed+ cells in both experiments suggests a second mechanism, herein termed material transfer, which can also result in the labelling of host photoreceptors with a genetic marker derived from the donor cells.
**Figure 2** | Host and donor photoreceptor exchange fluorescent reporter proteins as assessed by confocal imaging. NrlGFP post-mitotic photoreceptor precursor donor cells (green) were transplanted into DsRed (red) hosts and examined by confocal microscopy 5-6 weeks post transplantation. Nuclei are labelled with Hoechst (blue). (a, b), histograms showing quantification of the proportion of cells analysed (n = 157 cells; N = 5 retinae) that expressed GFP alone (GFP⁺) or GFP and DsRed (GFP⁺/DsRed⁺) when measuring inner segments (a) or cell bodies (b). (c), confocal projection image of a representative host retina. Lines represent regions of interest (ROIs) through cells and inner segments shown in (d-g). (d), (e) single confocal sections and the respective line plots through two inner segments and (f, g), two cell bodies shown in (c). (h, i), scatter plot (mean ± s.d.) and histogram showing the position of GFP⁺ and GFP⁺/DsRed⁺ photoreceptors with respect to the outer limiting membrane (OLM) of host retina. The relative positions of the two populations were significantly different (P = 0.0486 two tailed t-test with Welch’s correction; P<0.001 2-way ANOVA). (j), confocal projection image showing donor NrlGFP⁺ cells in SRS of DsRed⁺ host. Arrows, donor GFP⁺ cells that are DsRed⁺. Scale bars 5 μm (c), 3 μm (d) and 10 μm (g).
Material transfer does not involve nuclear fusion. Transfer of genetic information is typically associated with cell fusion, either with or without nuclear fusion. Previously, we, and others, reported that integrated cells only ever contain a single nucleus\textsuperscript{9,10} and do so from the earliest stages examined post transplantation (48 h) \textsuperscript{[27]}. Here, we sought to re-examine the possibility of nuclear fusion for those host cells expressing donor-derived reporters. We examined 4\textsuperscript{1,000} GFP\textsuperscript{+} photoreceptors located within the ONL of transplanted wild-type or Gnat1\textsuperscript{−/−} host retinae (N = 6) stained for the nuclear envelope protein, LaminB\textsuperscript{35}. Without exception, all GFP\textsuperscript{+} cells had a single nuclear envelope and no evidence of polyploidy (Fig. 4a–c). The vast majority had highly condensed nuclei, typical of rod photoreceptors (>98%) \textsuperscript{[36]}, although cone-like profiles with multi-chromocentred single nuclei were seen occasionally.

A retrospective examination of previously published data\textsuperscript{27} confirmed that integrated GFP\textsuperscript{+} cells only ever contained a single nucleus from as early as 48 h post transplantation (n = 159 cells; N = 5 at 48 h post transplantation), arguing strongly against nuclear fusion, an event rarely seen between post-mitotic neurons\textsuperscript{23}. To rule this out conclusively, we transplanted male NrlGFP\textsuperscript{+} cells into female wild-type hosts and performed Fluorescent In Situ Hybridization (FISH) against the Y-chromosome, at 5–6 weeks post transplantation (Fig. 4d–g). Y-chromosome probe staining was detected in 83 (±7)% of photoreceptors in male NrlGFP eyes (positive control; Fig. 4d; N = 4), compared with 0 (±0)% of cells in female NrlGFP eyes (negative control; Fig. 4e; N = 3). Positive labelling for the Y-chromosome was detected in 84 (±10)% of donor cells within the sub-retinally located cell mass in transplanted eyes (N = 8).
We also detected rare examples of Y-chromosome labelling in GFP⁺ cells located within the recipient ONL (Fig. 4f). However, Y-chromosome staining was absent from the large majority of GFP⁺ cells located within the ONL (Fig. 4g and Supplementary Fig. 2).

The presence of Y-chromosome⁺ cells within the host retina, albeit in low numbers, together with previous observations in which donor cells with pre-labelled nuclei were present within host retina⁸ supports the occurrence of true donor cell integration. However, the marked difference between the number of GFP⁺/Y-chromosome⁺ donor cells within the SRS and the number of GFP⁺/Y-chromosome⁻ within the host ONL suggests that a significant proportion of GFP⁺ cells within the host retina arise by material transfer via a mechanism that does not involve transfer of the donor cell nucleus.

**Donor and host photoreceptors exchange array of gene products.**

The data above suggest that host photoreceptors can acquire reporter proteins that are genetically present only in the donor cell. We next considered whether this was a phenomenon particular to the fluorescent reporters used, or represents a more widespread mechanism. It has previously been shown that, in addition to the donor-derived fluorescent reporters, host cells display a variety of proteins that are otherwise missing from photoreceptors cells within the host ONL: for example, the photopigment rhodopsin is present in GFP⁺ cells within the ONL of Rho⁻/⁻ mice⁹,¹¹,¹³,¹⁹, the structural protein Peripherin is found in GFP⁺ cells in the Prph2⁰²²/²² ONL⁹,¹³,¹⁹ and rod α-transducin (encoded by the Gnat1 gene) is present in GFP⁺ cells in the Gnat1⁻/⁻ host retina¹², each one in its correct location and for many weeks post transplantation²⁷. Indeed, the presence of GFP⁺ cells in the host ONL has been observed as late as 1 year post transplantation³⁷.

We sought to determine how robust the apparent material transfer between donor and recipient cells is. We examined Gnat1⁻/⁻ recipient mice that had received either only NrlGFP⁺ donor cells or a mix of NrlGFP⁺ donor cells and DsRed⁺ donor cells (Fig. 5). By 6 weeks post transplantation, rod α-transducin, the protein absent from host photoreceptors, was found in >83% of GFP⁺ cells located within the recipient Gnat1⁻/⁻ ONL (Fig. 5a–c; n = 138; N = 4). These included isolated cells lying some significant distance away from the donor cells in the SRS (Fig. 5b). Importantly, this held true for both GFP⁺ and DsRed⁺ cells (Fig. 5f). In these experiments, based on the data presented above, we assume that a significant proportion of the GFP⁺ cells located within the ONL are host, rather than donor, cells. Occasionally, host ONL cells were rod α-transducin⁺ but GFP⁻ (Fig. 5d). Altogether with previously published data⁹,¹¹,¹³,¹⁹, this demonstrates that the mechanism leading to material transfer between donor and host cells is sufficiently robust to result in the presence of a wide variety of donor-derived proteins, including many that are genetically missing from diseased host photoreceptors.
Material transfer does not involve uptake of free proteins. We considered the possibility that free protein could be released into the environment by the transplanted donor cells that remain in the SRS and/or by resident macrophages and taken up by the host photoreceptors. Recombinant eGFP (rE GFP) was injected into the SRS of NrlGFP post-mitotic photoreceptor precursor donor cells were transplanted into adult Gnat1+/− hosts and immunostained for rod α-transducin 5–6 weeks post-transplantation (a–c). Confocal projection images of GFP+ cells (green) in the ONL of host retinas stained for rod α-transducin (red). Nuclei were stained with Hoechst 33342 (blue). Note that in (b) the right hand panel shows the same image but with increased gain for the blue channel to show the nuclei of the RPE: No donor cells were present in the SRS overlying the GFP+ rod α-transducin+ cells. (d). Examples of GFP+/rod α-transducin+ host cells (arrow) were also seen. (e), Histogram showing the mean percentage (±s.d.) of GFP+ photoreceptors within the host ONL that were rod α-transducin+ (n = 138 cells; N = 4 retinas). f, rod α-transducin staining was seen in the outer segments of both GFP- and DsRed-reporter-labelled photoreceptors (arrow). Scale bars 10 μm.

Altogether, these data indicate that material transfer to photoreceptors in the host ONL is a specific property of stage-specific donor photoreceptor precursors (and not other cell types) and does not result from uptake of free-floating protein.

Material transfer involves donor and host cell interaction. The use of rE GFP suggests that material transfer is not the result of uptake of free-floating GFP protein by host photoreceptors.

Transplantation of GFP+ fibroblasts induced macrophage infiltration and rejection of the transplanted cells in all eyes examined at 2 (N = 4) and 6 (N = 8) weeks post transplantation. Although autofluorescent macrophages were present, on no occasion was GFP signal found within the recipient ONL (Fig. 6g,h).

We have previously reported that transplanted GFP+ retinal progenitor cells (RPCs) survive in the SRS but do not integrate into the host retina2. We repeated this experiment, this time transplanting E11.5 cba.GFP+/− RPCs, prepared in the same way as postnatally derived donors, into adult Gnat1+/− recipients. Donor cells survived in the SRS, but in 3 out of 4 eyes examined, no GFP+ cells were found in the recipient ONL and just 3 were found in the ONL of the fourth eye (0.75 cells ± 1.5; N = 4) at 6 weeks post transplantation (Fig. 6i,j).

Figure 5 | Material transfer permits robust exchange of an array of gene products including those genetically absent from host photoreceptors. NrlGFP post-mitotic photoreceptor precursor donor cells were transplanted into adult Gnat1−/− hosts and immunostained for rod α-transducin 5–6 weeks post-transplantation (a–c). Confocal projection images of GFP+ cells (green) in the ONL of host retinas stained for rod α-transducin (red). Nuclei were stained with Hoechst 33342 (blue). Note that in (b) the right hand panel shows the same image but with increased gain for the blue channel to show the nuclei of the RPE: No donor cells were present in the SRS overlying the GFP+ rod α-transducin+ cells. (d). Examples of GFP+/rod α-transducin+ host cells (arrow) were also seen. (e), Histogram showing the mean percentage (±s.d.) of GFP+ photoreceptors within the host ONL that were rod α-transducin+ (n = 138 cells; N = 4 retinas). f, rod α-transducin staining was seen in the outer segments of both GFP- and DsRed-reporter-labelled photoreceptors (arrow). Scale bars 10 μm.
We next compared the distribution of labelled cells following transplantation of a mixed population of donor photoreceptor precursors taken from both NrlGFP and DsRed mice. Post-transplantation, GFP\textsuperscript{+} and DsRed\textsuperscript{+} cells were randomly distributed within the host ONL (Fig. 7a,b). Strikingly, we observed examples where both GFP and DsRed were localized in a single photoreceptor within the host ONL (Fig. 7c). These were rare in number (1.9% of all cells examined; $n = 500$, $N = 10$), but a similar pattern was also observed in donor cells that remained within the SRS. The majority of the donor cells in the SRS were either GFP\textsuperscript{+} or DsRed\textsuperscript{+}, but 6.0% of cells ($n = 300$, $N = 10$) colocalised both fluorescent signals (Fig. 7d,e). In all cases, labelled cells bore a single nucleus. The presence of dual-labelled cells within the host retina might be explained either by material transfer leading to the exchange of GFP and DsRed RNA, and/or protein between donor cells before one cell subsequently integrating, or by RNA and/or protein from both DsRed donors and NrlGFP donors being passed to a host photoreceptor cell.

The presence of even rare examples of dual-labelled cells is surprising. The observation that the majority of labelled cells were discrete entities, labelled with GFP or DsRed and located in the ONL, combined with the absence of fluorescence in the extracellular matrix, suggests that material transfer occurs specifically between donor and host photoreceptors. This could be via a direct physical cell–cell interaction or indirectly, by extracellular trafficking of (presumably packaged) RNA and/or protein between cells. The relatively low number of reporter-labelled host photoreceptors, compared with the total number of host photoreceptors, indicates either that relatively few host cells can undergo material transfer or that there is limited/transient supply of that material.

**Material transfer does not require sustained cell–cell fusion.** Recent studies have indicated that neural stem cells, among others, can fuse with adult neurons in a process that requires microglia\textsuperscript{25}. In these studies, careful examination of the adult neuron always revealed the presence of a second, reporter-positive cell adjacent to it. It is possible that material transfer similarly results from the direct physical interaction between donor and host photoreceptors. Examination of retinal sections from transplanted eyes shows that many GFP\textsuperscript{+} cells within the host ONL lie immediately below the donor cell mass (Fig. 8a). Moreover, their outer segments, by default, often terminate at its edge suggesting a potential physical interaction. However, numerous examples could be found of GFP\textsuperscript{+} cells located in
the host ONL without any GFP+ donor cells within the overlying SRS (Fig. 8b–d). When examined using 3D reconstruction, these cells had near-normal photoreceptor morphology with no evidence of physical attachment to a second cell (Fig. 8c,d). These data do not rule out the occurrence of transient physical interactions, but do indicate that a sustained physical connection between donor and host photoreceptor is not essential for material transfer.

Material transfer is widespread transient and repeated. The Cre/LoxP system has been used in a number of transplantation paradigms to investigate the occurrence of cell fusion. Here, we transplanted Crx.GFP+ ESC-derived photoreceptor precursor donor cells, which had previously been transduced with an ShH10.CMV.iCre gene therapy vector, into tdTomato+ reporter mice (Fig. 9). When cells expressing tdTomato+ acquire Cre recombinase, the LoxP sites are cleaved and the stop signal is excised allowing transcription of tdTomato. Because the signal is inducible, the incidence of false-positive signals should be low and fusion-type events would be seen as GFP+/tdTomato+.

Subretinal injection of ShH10.CMV.iCre virus into tdTomato+ mice confirmed the inducible expression of tdTomato (Fig. 9b). Conversely, no expression of tdTomato was observed in uninjected mice (Fig. 9c) or following subretinal injection of vehicle (Fig. 9d). To control for viral carry over from the donor cell preparation, the supernatant from the final cell wash was injected sub-retinally, as described previously19,40. Only a very small number of tdTomato+ cells (22 ± 20 photoreceptors per eye; N = 4), and no GFP+ cells, were observed in each retina (Fig. 9e). This last observation underlines the point that material transfer is highly unlikely to be the result of uptake by the host cells of free nucleic acid released by donor cells damaged during the preparation process.

We next transplanted Cre-expressing CrxGFP+ donor cells; an average of 2,119 ± 1,575 GFP+ cells (N = 5) were found within
Figure 9 | Transplantation of Cre+ photoreceptor precursors leads to widespread expression of floxed reporters in host cells. (a), box (25–75% percentile) and whiskers (min/max) plot showing median (line) number of reporter-labelled photoreceptors in adult dTomato+ host retinae following transplantation. **P<0.01 2-way ANOVA. N.B. all controls (uninjected, PBS-injected and final wash) were highly significantly different from both Cre+/GFP+ and TdTom+ populations but are not denoted on graph for clarity. (b-d), confocal projection images showing retinal sections after sub-retinal injection of (b) ShH10.CMV.iCre (5×10^12 viral particles per ml), (c) uninjected, (d) PBS-injected, or (e) mice injected with final wash from cell preparation. (f), confocal projection images of GFP+ (green) and tdTomato+ (red) cells within the floxed tdTomato host retinae following transplantation of Crx.GFP+/Cre+ donor cells. Note that there are significantly higher numbers of tdTomato+ cells than GFP+ cells. Inset, example of a GFP+, tdTomato+ cells, surrounded by GFP-/tdTomato+ cells. (g), Immunostaining for CRE (grey) shows robust protein expression in donor cells in SRS and some weaker expression in reporter-labelled cells within host retina. (h), some cells in the SRS cell mass were tdTomato+ (arrow), indicating the potential for bidirectional material transfer. For panels (f) and (g), images from top to bottom show individual and/or combined channels for the same region of interest. (i), tdTomato+ cells were present in areas where donor cell masses were present but no GFP+ cells within the underlying host ONL (j), few, if any, GFP+ or tdTomato+ cells were seen in retinae where donor cell mass had been rejected (N = 2 retinae). Scale bars 10 μm.
the recipient ONL, a level similar to that found using this donor line (without prior transduction with ShH10.CMV.iCre virus) into Gnat1\(^{-/-}\) hosts (2,698 ± 591 GFP\(^{+}\) cells; \(N = 7\)). Many of these cells also expressed tdTomato (Fig. 9g), indicating that they were host photoreceptors that received Cre Recombinase by material transfer. Note that Cre was introduced into donor cells using a virus and so not all GFP\(^{+}\)/tdTomato donor cells would have been transduced. The presence of GFP\(^{+}\)/tdTomato\(^{-}\) cells within the recipient ONL therefore reflect either true integration events or material transfer from a GFP\(^{+}\)/tdTomato\(^{-}\) donor cell. In addition, we also observed small numbers of GFP\(^{+}\)/tdTomato\(^{-}\) cells within the cell mass in the SRS (Fig. 9h). This provides further evidence that material transfer can also occur from host to donor, although this appears to be less frequent.

Strikingly, in addition to GFP\(^{+}\)/tdTomato\(^{-}\) cells, we observed 18 times more photoreceptors that express tdTomato alone (GFP\(^{-}\)/tdTomato\(^{+}\); 39,755 ± 9,888 cells) (Fig. 9a,f,g). TdTomato\(^{+}\) cells were frequently seen in regions immediately below the donor cell mass, even in the absence of GFP\(^{+}\)/tdTomato\(^{-}\) cells in the host retina (Fig. 9g). This is highly unlikely to be due to viral carry over: (i) injection of final wash led to little, if any, labelling of host cells (Fig. 9e), (ii) staining for CRE protein showed robust labelling of the majority of donor cells in the SRS and some, but not all, tdTomato\(^{+}\) cells in the host ONL were CRE\(^{+}\) but expression was weaker than in donor cells in the SRS (Fig. 9g) (all tdTomato\(^{+}\) cells would be expected to be robustly CRE\(^{+}\) if virally-transduced), (iii) transplantation of ESC-derived precursors labelled with the same capsid (ShH10.Rhop.eGFP), led to 1,745 ± 318.7 (\(N = 9\)) reporter-labelled cells in the host retina, very similar to that seen for Cre.GFP ESC-derived photoreceptors and more than a magnitude lower than the number of tdTomato\(^{+}\) cells seen and (iv) tdTomato labelling was absent in eyes in which the cell mass had been rejected (Fig. 9j; \(N = 2\)).

That so many more cells in the host retina were GFP\(^{-}\)/tdTomato\(^{+}\) than GFP\(^{+}\)/tdTomato\(^{-}\) suggests that material transfer is a widespread phenomenon, but may not be sustained in all cells at levels sufficient to maintain expression of proteins such as GFP. Conversely, once sufficient levels of Cre Recombinase are present and Cre recombination occurs, the tdTomato reporter will be expressed permanently. It therefore reflects all previous recombination events, rather than the number of material transfer events occurring at that point in time.

**Discussion**

Rescue of retinal degeneration by the transplantation of healthy photoreceptors has received considerable interest in recent years. Numerous studies have reported that transplantation of reporter-labelled donor photoreceptor cells, typically obtained from developmental stages equivalent to the post-mitotic precursor stage, into the adult wild-type and diseased retina leads to the presence of reporter-labelled cells in the host retina\(^a,12-14,19,27,37,41,42\). These apparently integrated photoreceptor cells were understood to arise from their migration into and integration within the host retina. Here, we present data to show that while donor cell migration and integration occurs, it reflects only a small proportion of events leading to the presence of donor-reporter-labelled cells within the host retina. Instead, we propose that post-mitotic donor and host photoreceptors engage in the transfer of cellular material, either as RNA and/or proteins, resulting in a wide variety of donor-derived proteins, including structural ones such as peripherin-2 and rhodopsin\(^9,11,13,19\), being present in host photoreceptors.

This process of material transfer accounts for the majority of reporter-labelled cells within the host retina and raises the need to re-evaluate the cellular mechanisms underlying photoreceptor transplantation and their relative contributions to rescue of retinal degeneration. Almost all reporter-labelled cells in the host retina were additionally immunopositive for proteins otherwise expressed only by donor cells (for example, rod\(\alpha\)-transducin in Gnat1\(^{-/-}\) recipients\(^12,27\); Peripherin-2 in Pph2\(^{12,42/42}\) recipients\(^13,19\)), which indicates that material transfer is a robust process that renders the majority of host cells undergoing material transfer functional, at least for a period of time. However, rather than integrating, the donor cells appear to be able to engage in transfer of a broad spectrum of gene products, through the transfer of RNA and/or proteins.

The cellular mechanisms underlying material transfer remain to be determined and will likely need to account for an extraordinary number of experimental findings, past and present. The mechanism does not involve classic nuclear fusion, as indicated by FISH, or the transfer of free protein or free nucleic acid. It does not require sustained physical interactions between donor and host photoreceptors, as demonstrated by the frequent presence of isolated GFP\(^{+}\)/rod\(\alpha\)-transducin\(^{+}\) cells in the host Gnat1\(^{-/-}\) retina with no donor cells in the overlying SRS. Similarly, 3D reconstructions of such cells show only a single cell, with no evidence of cell–cell fusion or sustained physical contact. The Cre/LoxP experiments lend further evidence in support of this; the number of tdTomato\(^{+}\) cells within the host retina far exceeds that of GFP\(^{+}\) cells. Host cells bearing floxed tdTomato sites will require Cre Recombinase at a single moment in time to undergo recombination and permanently express tdTomato. Conversely, GFP’s half-life\(^38\) means that host photoreceptors would require a near-constant supply of GFP mRNA or protein to express it at levels sufficient to be detected by fluorescence imaging (or flow cytometry). Our data indicates a mechanism whereby host cells receive donor cell-derived material in a transient, but frequent, manner. The duration of rescue of any particular gene or protein is likely to be influenced by the long-term viability of the donor cell mass and true integrated cells as well as the half-life and turnover of the protein in question. The mechanism is a feature specific to photoreceptor precursor donor cells, rather than more immature cells, in accordance to previous reports\(^9,14,19\), and does not appear to involve the release of either proteins or nucleic acid into the extracellular milieu.

Finally, the mechanism must also explain a wide range of data that previously supported the notion of donor cell integration, including the ability to manipulate the number of donor-reporter-labelled cells within the host by altering structural aspects of the host retina such as OLM integrity\(^13,38,29\) and chondroitin sulphate proteoglycan deposition\(^13,45,44\) and by improving donor cell survival using the overexpression of growth factors\(^45\).

The majority of reports describing fusion-type events have typically involved the fusion of a stem cell with a differentiated cell and the acquisition of the stem cell’s nucleus by the host cell and formation of a bi-nucleated cell\(^40\), a feature that was never observed here. Intriguingly, however, hematopoietic stem cells have been proposed to contribute genetic information in a transient manner to Purkinje cells. Nern et al.\(^46\), proposed that nucleic acid and/or protein transfer between hematopoietic cells and Purkinje cells occurred either through transient fusion or intercellular vesicular transport mechanisms. Indeed, the vesicular transfer of DNA, mRNA and even organelles from bone marrow-derived cells to various different tissues has been reported recently\(^47\) and may represent an important method of tissue repair. Most recently, exosomes, a type of microvesicle, have been described to mediate the transfer of mRNA, miRNA and protein from oligodendrocytes to neurons in the undamaged brain in an activity-dependent manner\(^48\). At the present time, it is
not possible to conclude whether material transfer between donor and host photoreceptors primarily involves the exchange of nucleic acid or protein, or even a combination of the two. While we can reasonably reject the notion that it involves the uptake of either free protein or free nucleic acid, it is tempting to speculate that microvesicles, or other structures that can similarly package nucleic acid and proteins, might play a role in material transfer between donor and host photoreceptors.

This study shows that material transfer must now be taken into account when designing applications for clinical translation. If the underlying cellular mechanisms of material transfer can be elucidated, they represent a novel therapeutic approach for introducing functional proteins into otherwise diseased photoreceptors, equivalent to a broad-spectrum gene-replacement approach. It will be particularly interesting to determine if human photoreceptor precursors can engage in material transfer. More immediately, however, cell-replacement therapy is most likely to be utilized in end-stage disease where there are very few photoreceptors remaining in the area of transplantation. It will be particularly important to determine the extent to which this function can be rescued by the transplantation of human photoreceptor precursors. When elucidated, they represent a novel therapeutic approach for retinal degenerations, equivalent to a broad-spectrum gene-replacement approach. 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A volume of 1 μl of the cell suspension was injected sub-retinally, as described above.

**Preparation of rEGFP.** rEGFP purified from E. coli was reconstituted in sterile PBS at 1 mg ml⁻¹. A volume of 1 μl was injected into the SRS, as described above.

**Cell counts.** Eyes were collected 5–6 weeks post transplantation, unless otherwise stated, and cryosectioned before sectioning and mounting. GFP⁺ and DsRed⁺ cells were located using epifluorescence illumination. The average number of reporter-labelled cells per eye was determined by counting every third section and multiplying by three to give a total/eye, as described previously. The majority of reporter labelled cells per eye was determined by counting every third section and

Preparation of rEGFP

A volume of 1 μl of Protease K solution for 20 min, at 37°C. Eyes were collected 5–6 weeks post transplantation, unless otherwise stated. Individual xy images were acquired using a 2-frame time-lapse image stack series set to 37°C and 5% CO₂. For image acquisition, x2t time-lapse image stack series were captured at a resolution of 512 × 512, at a step size of 1 μm and at 20 min intervals.

**Statistical analysis.** All means are presented ±s.d., unless otherwise stated; N, number of retinae; n, number of cells. All transplantation cell count data is based on at least two independent transplantation runs. Each run therefore involves independent littermates of donor animals, cell preparation and FACS sorting, surgery and different litters of host animals. D’Agostino and Pearson’s test was used to determine normality of data sets, and the appropriate statistical tests applied, as required. The statistical program used for analysis was GraphPad Instat 3 (GraphPad Software, Inc., La Jolla, USA).

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding author on reasonable request.

**References**


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experimental design and viral constructs, and contributed to manuscript preparation. Y.D. contributed to histological processing and surgery. A.N. and M.K. contributed to the maintenance of stem cell cultures and viral production. E.C. contributed to viral construct design and production. K.W-C. contributed a data set for secondary analysis. J.C.S. contributed to the interpretation of experiments, manuscript writing and funding. A.J.S. contributed to the conception, design and interpretation of experiments, and manuscript writing. R.R.A. contributed to the conception, design and interpretation of experiments, manuscript writing and funding.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Pearson, R. A. et al. Donor and host photoreceptors engage in material transfer following transplantation of post-mitotic photoreceptor precursors. Nat. Commun. 7, 13029 doi: 10.1038/ncomms13029 (2016).

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