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Review

Pluripotent stem cell-derived retinal organoid/cells for retinal regeneration therapies: A review☆

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ABSTRACT

In recent decades, many researchers have attempted to restore vision via transplantation of retina/retinal cells in eyes with retinal degeneration. The advent of induced pluripotent stem cells (iPSC) and retinal organoid induction technologies has boosted research on retinal regeneration therapy. Although the recognition of functional integration of graft photoreceptor cells in the host retina from 2006 has been disputed a decade later by the newly evidenced phenomenon denoted as “material transfer,” several reports support possible reconstruction of the host-graft network in the retinas of both end-stage degeneration and in progressing degeneration cases. Based on proof of concept (POC) studies in animal models, a clinical study was conducted in Kobe, Japan in 2020 and showed the feasibility of cell-based therapy using iPSC retinal organoid technology. Although the graft potency of human embryonic stem (ES)/iPS cell-derived retinal organoid/retinal cells has been suggested by previous studies, much is still unknown regarding host capability, that is, how long-standing human degenerating retinas are capable of rewiring with transplanted cells. This review summarizes past POC studies on photoreceptor replacement therapy and introduces some new challenges to maximize the possible efficacy in future human clinical studies of regenerative therapy.

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Abbreviations: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; RP, retinitis pigmentosa; ONL, outer nuclear layer; MEA, multiple electrode array; MHC, major histocompatibility complex; OCT, optical coherent tomography; RGC, retinal ganglion cell; LGIR, lymphocyte graft immune reaction.

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1. Introduction

Retinitis pigmentosa (RP) is a group of diseases characterized by the primary loss of rod photoreceptor cells owing to their genetic backgrounds. The typical initial symptoms are night blindness and progressive loss of the peripheral visual field, followed by loss of

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central vision as secondary loss of cone photoreceptor cells in the central retina and macula occurs. The cause of the secondary death of cone photoreceptor cells is partly due to the decrease in rod derived cone viability factor (RdCVF) secreted by rod photoreceptor cells [1]. To date, more than 70 causal genes have been reported for RP (RetNet, https://web.sph.uth.edu/RetNet/). Gene therapy is being developed for specific genes such as *RPE65* [2]. However, gene therapy is most effective when used in the early stages, before the substantial loss of photoreceptor cells. The optogenetic approach involves inducing light-responsive channels in the host's remaining retinal ganglion cells (RGCs), and a recent study showed partial visual recovery in a late-stage RP patient using this method [3]. Cell-based therapy is another promising approach that aims to replace lost photoreceptor cells.

Photoreceptor cells play a role in the initiation of visual processing by transmitting signals to the secondary retinal neurons and then to RGCs, which then output the signal to the brain. In RP, secondary neurons and RGCs still survive for some time, even after photoreceptor loss. Thus, replacement therapy for photoreceptor cells appears to be adequate for RP. In fact, transplantation of fetal retinas was investigated in the 1990s [4–6] with positive functional data obtained at the superior colliculus after transplantation in rats with end-stage retinal degeneration. Human clinical studies have also been conducted to show the safety of fetal retina transplants, with possible beneficial effects demonstrated in some cases [7,8]. However, the use of fetal retinas is limited and has ethical issues; thus, the mechanism of possible efficacy by retinal cell transplantation in clinical settings remains inconclusive.

In 2004, MacLaren et al. reported that transplanted photoreceptor precursor cells from the neonatal mouse retina could efficiently integrate into the host retina when the recipient retina had a photoreceptor layer or outer nuclear layer (ONL) [9]. The advent of induced pluripotent stem cell (iPSC) technology has further boosted research in the field of regenerative therapy [10]. Several years later, retinal organoid technology was reported [11–15], which further enhanced the expectations for stem cell-based retinal regeneration. Recent progress in embryonic stem (ES)/iPS cell-based retinal cell therapy for RP is described here, together with the recent challenges, including a clinical study using iPSC-derived retinal organoids (iPSC-retinas).

2. Prior research

2.1. Photoreceptor cell transplantation in the presence of host photoreceptors

Following the report by MacLaren et al., Pearson et al. reported that integrated graft mouse photoreceptor cells can restore visual function [16]. Several research groups have attempted to improve the reproducibility of this method and utilize it for therapeutic applications. However, in 2016, Pearson and other groups simultaneously reported that what they had reported as graft integration was mostly a result of material transfer from the graft cells to the remaining host photoreceptor cells, rather than the integration of graft photoreceptors in the ONL of the host retina [17–19]. From this point, the general consensus in the field of cell-based regenerative therapy is that end-stage retinal degeneration models with few remaining photoreceptor cells should be used to investigate host-graft visual network reconstruction.

Although material transfer can be a possible flaw in the interpretation of host-graft network reconstruction, from a clinical point of view, this can also be a part of the beneficial effect of photoreceptor transplantation, particularly when there are remaining photoreceptors that are not fully functional due to a decreased expression of or a lack of essential proteins. In addition to photoreceptor transplantation as a replacement therapy, it is still noteworthy that graft photoreceptor cells can also have a protective effect, partly by secreting beneficial factors such as RdCVF [1] and also as a source of material transfer of missing or reduced proteins.

2.2. Reconstruction of visual networks via transplantation in end-stage retinal degeneration models

Although the reports on material transfer questioned past relevant studies, some researchers have obtained proof of concept (POC) data using end-stage retinal degeneration models in which most of the photoreceptor cells were gone. In clinical settings, it is likely that end-stage RP would be the first target for regenerative therapies. Singh et al. showed the functional effect of transplantation of photoreceptors from postnatal day 3–4 mice using the pupillary test, behavior test, and cortical blood flow data [20]. Barnea-Cramer et al. transplanted human ES/iPS cell-derived photoreceptor precursors and showed improved visual function in behavioral tests [21]. In the same period, we also used end-stage retinal degeneration models for the transplantation of mice and human ES/iPS cell-derived retinal organoid sheets (ES/iPSC-retinas) [22–26].

Instead of using dissociated cells, the advantage of transplanting ES/iPSC-retinas may include the presence of neighboring retinal cells in the graft, such as Müller cells and horizontal cells, whose presence is considered important for photoreceptor functional maturation and synapse formation. For instance, Müller glia play a role in glutamate uptake and recycling of visual pigments for cone photoreceptors, and are considered important for the development of inner/outer segments [27,28]. Meanwhile, horizontal cells aid in the survival of photoreceptor cells, are components of the triad of photoreceptor ribbon synapses, aid in the invagination process of bipolar cells, and functionally contribute to the integration and regulation of the signals from photoreceptors [29,30]. Transplanted mice and human ES/iPSC-retinas at the retinal progenitor stage further develop after transplantation to form a photoreceptor layer, often in a rosette form.

We observed the morphological maturation of photoreceptor cells using immunohistochemistry and observed the inner/outer segment formation using electron microscopy, both by transplanting mice and human ES/iPSC-retinas in mice and immune-deficient mouse and rat retinal degeneration models, respectively (Fig. 1A and B) [22,23,25]. Host-graft synaptic contact was also observed using reconstructed 3-dimensional images of thick sections of transplanted retinas (Fig. 1C) [22,25]. We then introduced a multiple electrode array (MEA) system to sensitively record host RGC light responses in isolated transplanted retinas and confirmed that the RGC light responses in the grafted area were abolished by blocking the ON-bipolar pathway using the agonistic mGluR6 blocker L-AP4. These results indicated that these responses originated from photoreceptors, most likely from the graft (Fig. 2A–D) [24]. We also directly visualized host-graft synaptic contact using L7-GFP/rd1reporter host mice expressing GFP in their rod bipolar cells and ribeye reporter miPSC-retinas expressing tdTomato-CtBP2 at graft photoreceptor axon terminals, where we employed additional immunostaining of the post-synaptic marker Cacna1S (Fig. 2E and F). In our experiment, in behavioral tests employing a shuttle avoidance system to evaluate the learning capability of each mouse using beeps and lights, 35–40% of transplanted mice became responsive to light [24]. Following a similar strategy, we also showed the functional integration of human ESC/iPSC-retinas using MEA in immunodeficient retinal degeneration mice and rats (Fig. 3) [25,26]. McLellandt also reported maturation and functional integration of hESC-derived retinal organoids in nude

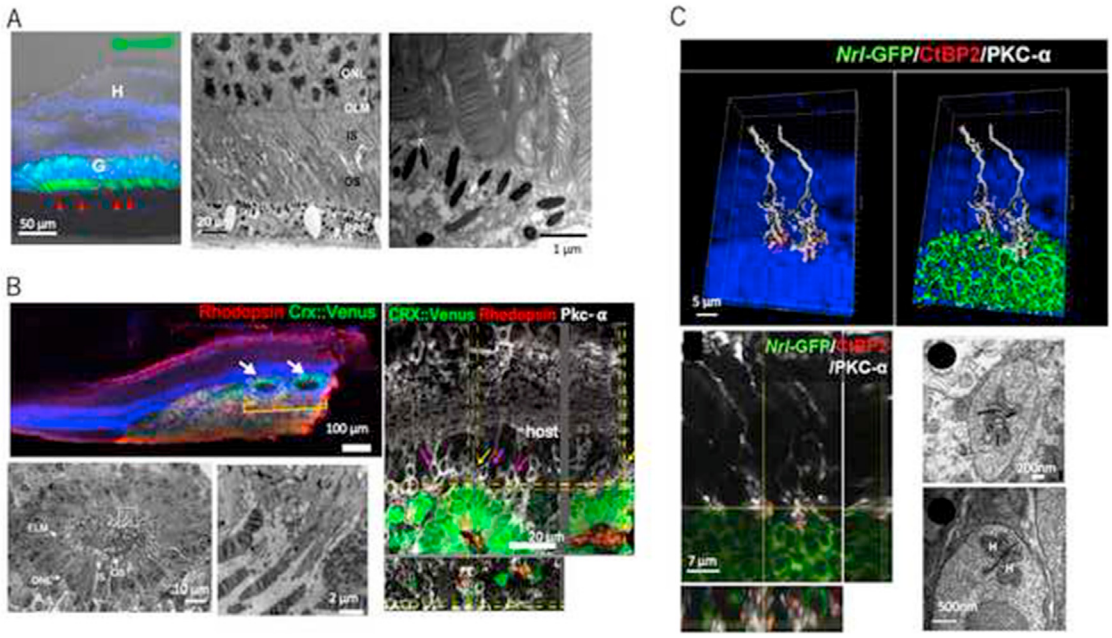

Fig. 1. Morphological maturation of ESC/iPSC-retinas after transplantation and host-graft synapse formation. A. Photoreceptor cells in mouse iPSC-retinas develop outer segments after transplantation in the end-stage degeneration mouse retina (*rd1*) (modified from Assawachananont J et al., Stem Cell Rep, 2014 [22]). B. Photoreceptor cells in human ESC-retinas develop outer segments in the end-stage degeneration rat retina (SD-Foxn1 Tg(S334ter)3Lav) and host-graft synaptic contact was observed (modified from Shirai et al., Proc Natl Acad Sci U S A. 2016 [23]). C. Host bipolar-graft photoreceptor synapses observed in the reconstructed 3-dimensional image. Graft photoreceptor cells express GFP under *Nrl*-promotor and presynaptic marker CtBP2 was localized at the host bipolar dendrite tips. Photoreceptor triad synapses (H:horizontal cells) were also observed at the host-graft contact site (modified from Assawachananont J et al., Stem Cell Rep, 2014 [22]).

rats with retinal degeneration using optokinetic response testing and visual responses from the superior colliculus [31].

More recently, isolated human ES/iPSC-derived cone cells were tested for functional integration. Ribeiro et al. labeled ES-derived cone photoreceptors by inducing L/Mopsin-driven GFP in differentiation week-13 cone photoreceptors, following which the sorted cells were transplanted into 3-month-old *rd1/Foxn1*^{nu} mice [32]. Using MEA, they showed that wild-type graft cone photoreceptors elicited host RGC light responses via the mGluR6 receptor-mediated pathway, whereas dysfunctional graft human cones (CNGB3) did not elicit RGC light responses. The study also showed that visually evoked behavior patterns were improved in human cone-transplanted mice.

Gasparini et al. challenged the transplantation of human cones in "ONL-remaining host retinas," which became relatively taboo after the report on "material transfer" [33]. They used human iPS cells expressing GFP under a cone-arrestin promoter and purified cones on differentiation day 200 for transplantation in Cpfl1 mice with remaining ONLs. The cells started to integrate 10 weeks after transplantation and were integrated further over 26 weeks. Full incorporation of graft cone cells was observed in 60% of the transplanted area 26 weeks after transplantation, and these photoreceptors developed into inner segments. They then showed synaptic contact using mGluR6 and ribeye immunostaining, as well as host RGC light responses via the mGluR6 receptor using MEA.

Aboualizadeh et al. transplanted photoreceptor cells from CRX$^{+/tdTomato}$ human ESCs between differentiation days 74–106 in eyes with laser-ablated monkey retinal degeneration. They observed neurite extension of graft photoreceptors through *in vivo* imaging using fluorescence adaptive optics scanning light ophthalmoscopy and histology [34]. These results indicate the potency of the functional integration of human ES/iPSC-derived photoreceptor cells. Additionally, although *in vitro* functional maturation of photoreceptor cells in retinal organoids has been considered difficult compared to *in vivo* maturation after transplantation, cone photoreceptor cells in retinal organoids have recently been shown to exhibit light responses comparable to adult primate foveal cones *in vitro* [35]. This may also indicate the competency of human ES/iPSC-derived photoreceptor cells for transplantation.

These animal studies indicate that restoration of the visual network by graft photoreceptor transplantation, either as a sheet from retinal organoids or with purified cells, is possible in end-stage retinal degeneration and in degenerating retinas (Fig. 4). For human cone photoreceptor cell transplantation, a clinically optimized purification strategy is required, which is partly suggested by the use of antibodies such as CD73 [36,37].

3. A clinical trial using iPSC-Retinas in RP patients

Although reporter animals and synaptic reporter lines are not available for studies using human ES/iPS cells, we obtained reproducibility using human ES/iPSC-retinas in Rho mutant retinal degeneration nude rats (SD-Foxn1 Tg(S334ter)3Lav) using immune histology and MEA [25,26] Additionally, the long-term survival of hESC/iPSC-retinas and maturation of graft photoreceptor cells was also confirmed in laser-induced focal retinal degeneration models of non-human primates [23,26] In one monkey, mild recovery of the visual field at the graft site was observed using a visually guided saccade test, in which the survival of graft photoreceptors was evident in the eye, with many of them positioned in the correct orientation in a semi-spherical rosette form after over 2 years (Fig. 5) [26].

For clinical application, a quality check protocol was created by our group at RIKEN BDR in collaboration with Sumitomo Pharma Co. Ltd [38], and a safety test was also conducted. The first human

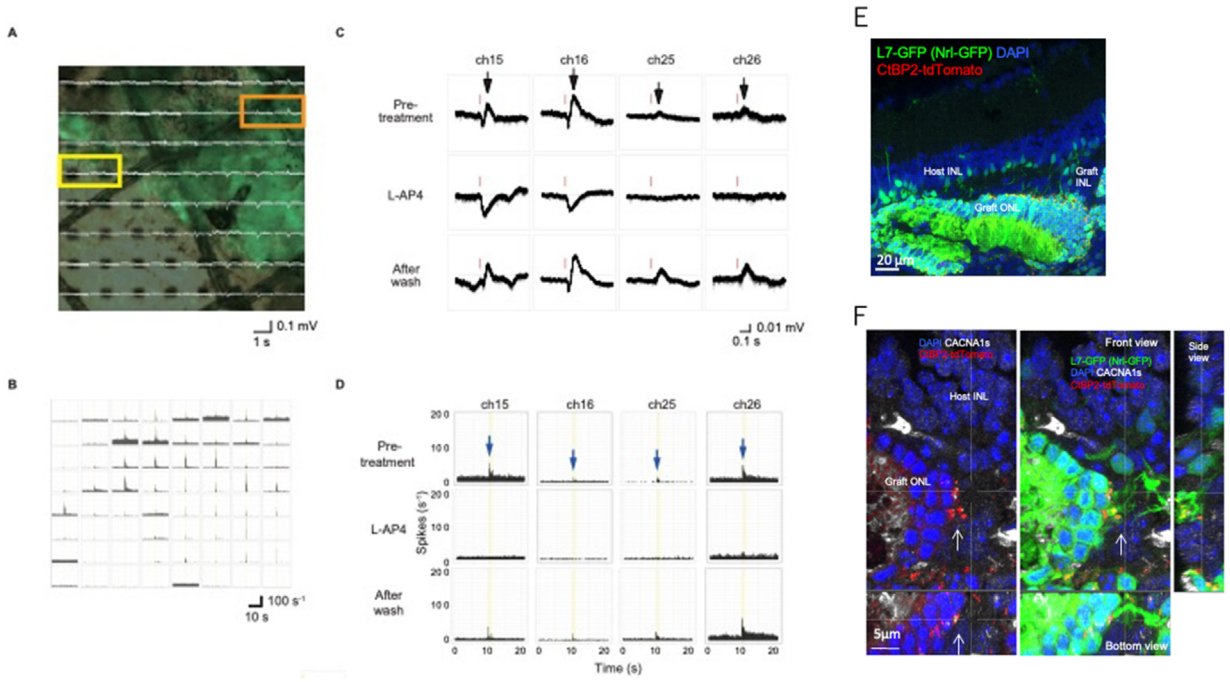

Fig. 2. Host retinal ganglion cells respond (RGC) to light after transplantation. A. After dark adaptation, a transplanted retina was isolated and placed with the RGC side attached on the electrodes using multiple electrode array (MEA) system. B. Peri-stimulus histogram on each electrode after spike sorting. C–D. Micro-Electroretinogram (ERG) wave patterns and RGC responses at the electrodes indicated by the orange (ch15, 16:on the graft) and yellow (ch 25, 26: edge of the graft) boxes. The responses were abolished by mGluR6 agonistic blocker LAP-4 and recovered after wash-out, indicating that these RGC responses were photoreceptor origin. E. GFP positive host bipolar cells (L7-GFP) extend dendrites into graft photoreceptor cells (Nrl-GFP) and contacted CtBP2-TdTomato expressed by graft photoreceptor cells under Nrl-promotor. F. Host bipolar cells contacted CtBP2-TdTomato expressed by graft photoreceptor cells where post-synaptic marker Cacna1S was also localized (modified from the publication by Mandai et al. Stem Cell Rep, 2017 [24]).

clinical study using hiPSC-retina organoids for advanced RP patients was approved in June 2020 and conducted at Kobe City Eye Hospital in Japan, supported by the Japan Agency for Medical Research and Development (AMED) and Sumitomo Pharma Co. Ltd. (jRCTa050200027). Three iPSC-retina sheets were transplanted in the eyes of two patients, and the graft survived stably for over 1 year in a severely advanced retinal degenerative environment without any serious adverse events. To avoid any possible risk of rejection, oral cyclosporine was administered for the first six months. Lymphocyte graft immune reaction (LGIR) tests that sensitively detected graft rejection in our previous clinical study [39] were also conducted. The proliferation of recipients' peripheral blood monocular cells (PBMCs) against retinal organoids from the same iPS cell line was evaluated, and it was confirmed that there was no graft rejection during or after the termination of cyclosporine administration.

Access to visual function in patients with ultralow vision is another issue in the evaluation of treatment efficacy. In addition to routine examinations, the square localization test, direction of motion test, full field stimulus test (FST), and alphabet recognition test were included in our clinical study. FST can evaluate the overall perception of the entire visual field, is used in the evaluation of gene therapies, and is considered a very sensitive test [40]. Results of other visual function tests should be reported soon.

4. Immunological aspects of hES/iPSC-retina transplantation

In addition to the eye being an immune-privileged site [41], previous clinical studies have reported a low risk of rejection of fetal retinas after transplantation [7,8]. More recently, Yamasaki et al. reported the low immunogenicity of human ES/iPSC retinas and their immunosuppressive nature, partly due to their secretion of TGF-β [42]. Uyama et al. reported a more detailed study using major histocompatibility complex (MHC)-matched and-mismatched transplantation in laser-induced retinal degeneration models of non-human primates [43]. There were no clinical signs of rejection, and the graft cells stably survived and matured following the MHC-mismatched transplantation, even after repeated transplantation without systemic immune suppression. These results indicate that transplantation to both eyes or multiple transplantation sessions may be clinically acceptable. However, LGIR tests implied subclinical rejection in three of four mismatched transplantations, while two MHC-matched transplantations did not show the same results. These monkeys with subclinical rejection also showed instances of strayed grafts in the choroid, possibly due to laser injury during model preparation and some transplantation artifacts, also implying a risk of evoking host immune responses when the MHC-mismatched graft is exposed to the host systemic immune system. Although stem cell-derived retinas may have a low risk of rejection, careful follow-up is also important for the long-term survival of MHC-mismatched transplants.

5. Future directions

The first clinical study at Kobe City Eye Hospital suggested the feasibility and safety of a regenerative therapeutic approach using pluripotent cell-derived retinal organoids, and good survival of the iPSC-retina sheets was confirmed in eyes with advanced retinal degeneration. However, each sheet measures approximately 0.5×1 mm, and the area covered by the graft is very small. Even if the grafted photoreceptor cells could make a successful synaptic connection with the host retinal cells, the improvement in visual function would be subtle. Thus, the next step would be to use grafts that cover a larger area by either using a greater number of sheets or a larger sheet.

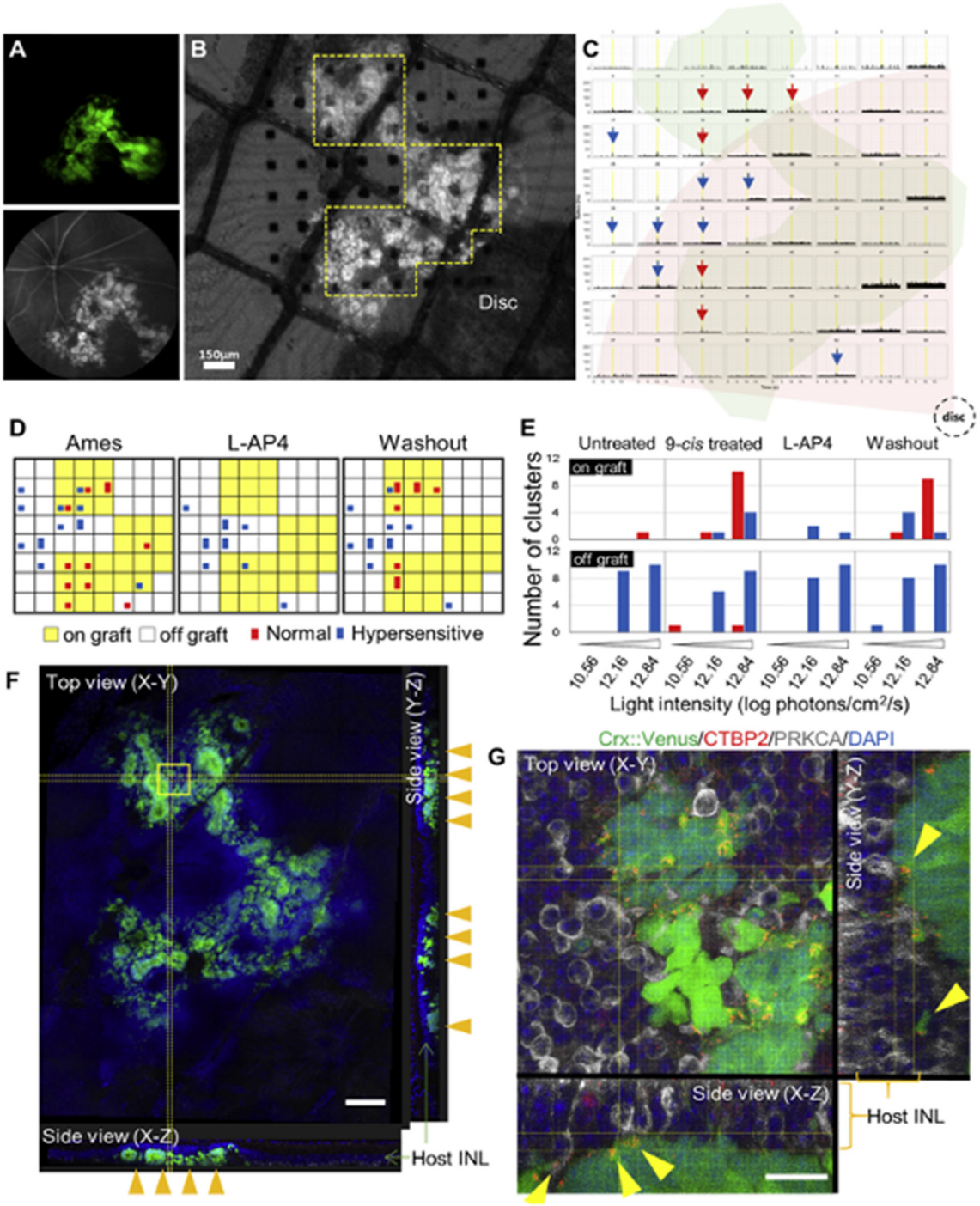

Fig. 3. Human ESC-retinas also make synaptic contact with host bipolar cells and elicit host RGC light responses. The isolated *NOG-rd1* retina after human ESC-retina transplantation expressing *CRX*::GFP. Light responses were recorded by multiple electrode array system and the presence of host-graft synapses were also suggested in the wholemount staining of the same retina (modified from Iraha et al., Stem Cell Rep, 2018 [25]).

One potential technical direction of stem cell-based therapy is combined treatment with gene modification. One major trend is to correct the causal gene in autologous iPS cell-derived retinas or retinal cells for transplantation [44]. In another approach, Garita-Hernandez et al. reported the induction of optogenetical tools in graft photoreceptors rather than in the remaining host retinal cells, such as host RGCs, as is routinely performed [45]. Photoreceptor cells utilize visual pigment opsins for light perception, and opsins require retinoid recycling, which is performed by retinal pigment epithelial (RPE) cells. However, after transplantation, graft cells are not always positioned correctly toward the RPE, or RPE cells can even be missing in advanced RP. Herein, optogenetically engineered photoreceptor cells are expected to function regardless of the absence of RPE and utilize an intra-retinal network.

Our attempt to enhance functional graft integration using ES/iPSC-retinas is to delete unwanted cells such as graft bipolar cells that may compete with host bipolar cells to form host-graft synapses, while retaining the photoreceptors and other supportive graft cells, including Müller cells and horizontal cells. For this

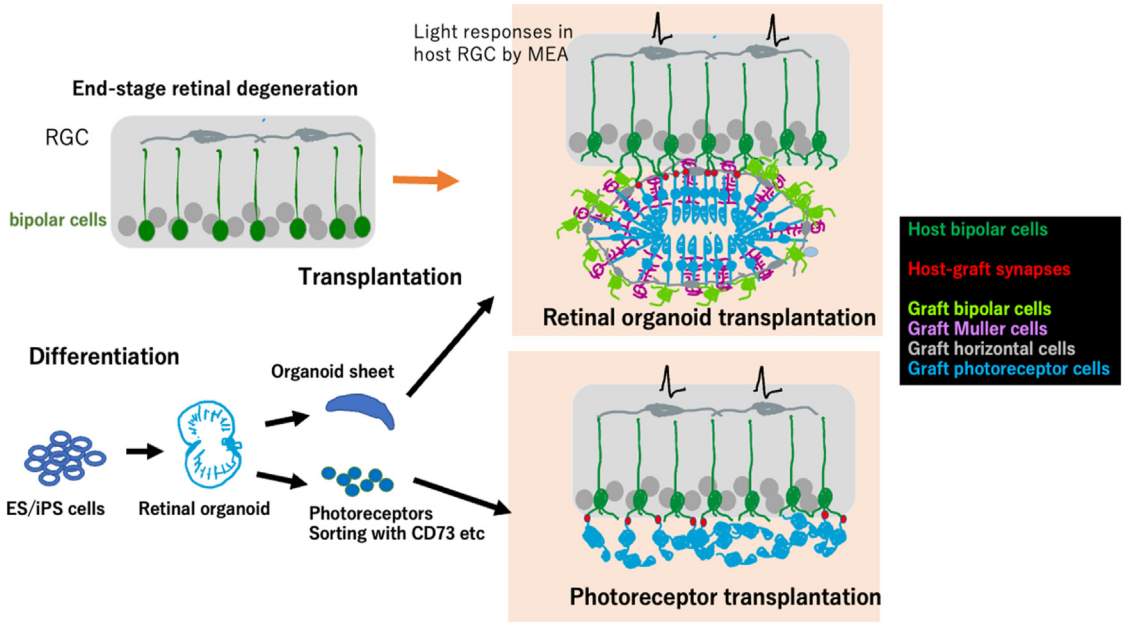

Fig. 4. Summary of ES/iPS cell-derived retina/retinal cell transplantation. Sheets or purified photoreceptors from retinal organoids have been shown to develop mature photoreceptor cells that make synaptic contact with host bipolar cells after transplantation in end-stage retinal degeneration models. Organoid sheets generally develop a photoreceptor layer in a rosette form together with graft Müller cells, bipolar cells, and horizontal cells after transplantation, and graft photoreceptors can develop outer segments to different degrees. Purified cone photoreceptor cells can also make synaptic contact with host bipolar cells. In both transplantation types, host RGC responses were obtained using a multiple electrode array system, which was blocked by the mGluR6 agonistic blocker L-AP4, indicating the response originated from photoreceptor cells.

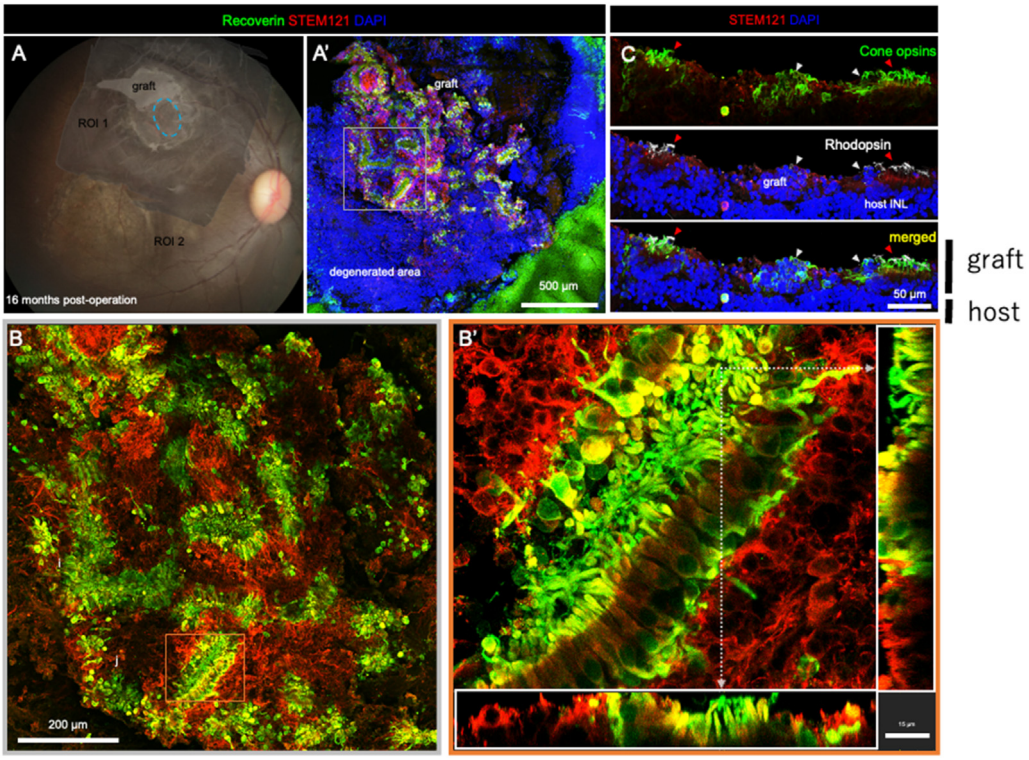

Fig. 5. Human iPSC-retina survived over 2 years in focal retinal degeneration model of non-human primate. A–B. In a retinal sample of over 2 years after transplantation, multiple STEM121(human marker)/recoverin-positive hemispherical graft photoreceptor rosettes were densely observed at transplanted site midst the area of host photoreceptor ablation (A′→B→B′; a sequential magnification of the boxed area). The graft photoreceptor cells show outer segment-like structures. C. Graft photoreceptor cells show localized expression of cone and rod opsins at outer segments in hemispherical open rosettes (modified from Tu and Watanabe et al., Stem Cell Rep, 2019 [26]).

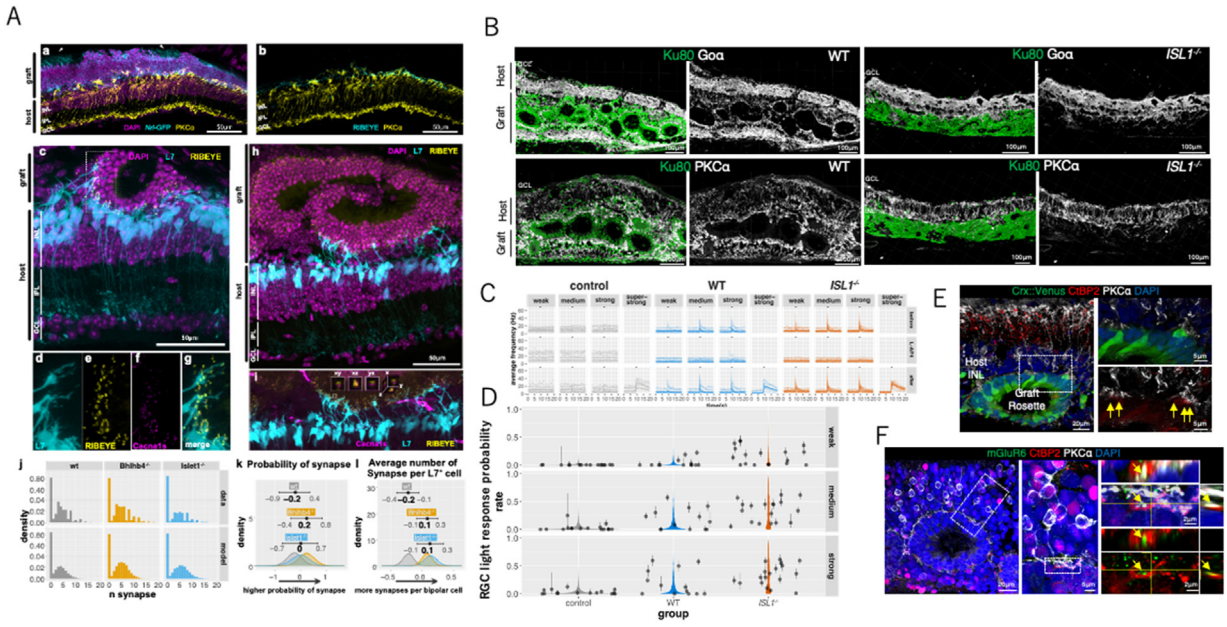

Fig. 6. Deletion of host bipolar cells enhance functional integration of ESC/iPSC-retinas. A. Host bipolar cells in rd1 mice robustly extend dendrites and contact RIBEYE positive photoreceptor synapses in mouse *Bhlh4*−/− iPSC-retinas that lacks rod bipolar cells (a–b). Host bipolar-graft synapse was quantitatively accessed in 3-dimensional analysis using L7-GFP/*rd1* reporter mice and CtBP2:TdTomato reporter mouse ESC-retinas (c-k). The number of synapses per GFP positive host bipolar cell increased in bipolar cell-deleted grafts (j–k). (Modified from Matsuyama et al., iScience, 2021 [46]). B. Deletion of *Islet1* gene also led to the loss of ON-bipolar cells in human ESC-retinas after transplantation in retinal degeneration rat (SD-Foxn1 Tg(S334ter)3Lav). C–D. Both wild type and *ISELT1*−/− human ESC-retinas elicited host RGC light responses after transplantation, but the light response probability was significantly higher with *ISL1*−/− grafts than with wild-type grafts. E–F. With the loss of graft bipolar cells, the host bipolar dendrite can be traced in detail and host-graft synaptic contact was observed with the presence of pre- and post-synaptic markers (yellow arrows). Right panels are magnification of the boxed area in the left panels (figures B–F are modified from Yamasaki et al, iScience, 2021 [47]).

purpose, we first deleted the genes involved in bipolar cell specification, such as *BHLHB4* or *ISLET1*, in mouse-ESC/iPSC-retinas [46]. The deletion of the *BHLHB4* or *ISLET1* gene consistently led to a marked loss of graft bipolar cells with increased amounts of host-graft synapses per host rod bipolar cell, which were analyzed in detail using L7-GFP/rd1 reporter mice with CtBP2-tdTomato reporter mESC-retinas and mGluR6 immunostaining (Fig. 6A). The rd1 mice transplanted with these bipolar-deprived grafts showed an improved response to light in behavioral tests. Furthermore, the similar trend was reproduced using human ESC-retinas with ISL-1 deletions [47]. Again, the loss of grafted ON-bipolar cells led to an increase in host-bipolar/graft-photoreceptor contact and increased host RGC responses to light by MEA (Fig. 6B–D). In this study, we identified possible host-graft synapses using various synaptic markers at the host bipolar cell dendrite tips in the absence of graft bipolar cells (Fig. 6E, F). By applying this technology to clinical therapy, we may be able to expect an increased ratio of patients who can benefit from ES/iPSC-retina transplantation. We are currently preparing a future clinical study that will utilize a clinical-grade *ISLET1* gene-deleted cell line.

Another area of interest may be *in vivo* imaging to assess graft cell function. Adaptive optic technology has enabled the observation of the spatial distribution of photoreceptor cells or photoreceptor inner segments [48], and adaptive optical coherence tomography can now visualize individual cone photoreceptor cells and Müller cells [49]. Phase-sensitive full-field swept-source optical coherence tomography (FF-SS-OCT) can capture osmotic effects on the outer segment of the eye, thereby allowing the observation of single cone activity in the human eye [50]. In addition, by using FF-SS-OCT on the eye of a healthy volunteer, simultaneous functional imaging of activation in the photoreceptor and ganglion cell layer/inner plexiform layer was acquired [51]. It would be exciting to apply such technology in the observation and evaluation of graft cells, their function, and graft-host signal transmission in regenerative therapy.

6. Summary

Regenerative therapy is an ideal treatment that many researchers wish to develop. New technologies, such as iPS cell induction and *in vitro* differentiation of retinal organoids, have motivated researchers to push regenerative research toward clinical applications. Despite phenomena such as the material transfer, evidence from multiple laboratories have accumulated, and now it seems that some kind of host-graft synaptic reconstruction is possible using pluripotent stem cell-derived retinal organoids or retinal cells in animal models. Notably, the first clinical trial using iPSC retinal organoids has already been safely conducted.

Although the clinical potency of iPS/ESC-retinas/retinal cells has been suggested by multiple studies, retinal network reconstruction requires simultaneous cooperation by the host. Considering that preclinical studies for cell therapies can only be investigated using xeno-transplantation or in animal models, whether long-standing human degenerating retinas can acquire plasticity and accept grafted retinal cells has yet to be elucidated. A further breakthrough may be required before host retinal cells robustly respond to graft cells. In addition, once the safety of ES/iPSC-retina/retinal cells has been assured through clinical studies, we may also gradually be able to determine suitable hosts for graft integration acceptance. Reconstruction of substantial vision may still be challenging; however, research on retinal regeneration is gradually but definitely progressing, with clinical studies beginning to emerge.

Declaration of competing interest

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