Stem Cell Biology and Regenerative Medicine

Marco A. Zarbin
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Ricardo P. Casaroli-Marano *Editors*

Cell-Based Therapy for Degenerative Retinal Disease

Humana Press

Stem Cell Biology and Regenerative Medicine

Series Editor

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Our understanding of stem cells has grown rapidly over the last decade. While the apparently tremendous therapeutic potential of stem cells has not yet been realized, their routine use in regeneration and restoration of tissue and organ function is greatly anticipated. To this end, many investigators continue to push the boundaries in areas such as the reprogramming, the stem cell niche, nanotechnology, biomimetics and 3D bioprinting, to name just a few. The objective of the volumes in the Stem Cell Biology and Regenerative Medicine series is to capture and consolidate these developments in a timely way. Each volume is thought-provoking in identifying problems, offering solutions, and providing ideas to excite further innovation in the stem cell and regenerative medicine fields.

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Preface

Cell-based therapy for ocular disease entered clinical practice in 1905 when the Austrian ophthalmologist, Eduard Konrad Zirm, performed the first successful corneal transplant. Many innovations in anterior segment cell-based therapy have followed, including stem cell transplants for ocular surface disease, which have their origin in the conjunctival transplants developed by Richard Thoft in 1977. The time course for cell-based therapy as a treatment for degenerative retinal disease has been much slower, in part due to biological challenges (e.g., developing appropriate donor tissue sources and the integration of donor and host tissue) as well as technical challenges (e.g., delivery of survivable tissue to the host and clinical grade protocols). Nonetheless, the eye is well suited to serve as a model for central nervous system cell-based therapy due to the current availability of safe and effective surgical delivery approaches, the partially immune suppressive nature of the posterior segment, the capacity to image in situ the transplanted tissue at high resolution, and the improved availability of donor tissue following the evolution of stem cell technology. Many important hurdles remain, and, in the pages that follow, these obstacles are reviewed in detail. Clinical features of target diseases, biological obstacles to donor-host integration, immune tolerance of the host, the latest retinal imaging technology, cell delivery technology, surgical approaches, clinical trials in progress, and regulatory issues are explored by thought leaders in each of these areas. This text will enable physicians, scientists, and industry experts to become acquainted with the state of the art in this rapidly progressing field. Within the next 5–10 years, a number of novel and promising technologies are likely to emerge that will accelerate the transition of cell-based therapy from laboratory experiments to approved clinical treatments of patients with blinding retinal disease, nearly all of whom currently lack an effective alternative for sight restoration.

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Chapter 1
Advantages of the Eye as a Target Organ for Cell-Based Therapy in the Central Nervous System

Marco A. Zarbin

Introduction

In principle, cell therapy can be used to treat ophthalmic metabolic abnormalities by supplying missing enzymes, needed neurotrophic agents, or inhibiting pathological processes such as choroidal neovascularization (CNV). In principle, cell therapy also can be used for ophthalmic regenerative medicine, which is sight-restoring and not just sight-preserving. Examples of the latter approach might include transplanting photoreceptors (PRs) to treat blindness due to retinitis pigmentosa (RP), a group of diseases in which PRs, the light-sensing cells of the retina, die, or transplanting ganglion cells, the output cells of the retina that connect via the optic nerve to the lateral geniculate nucleus, to treat glaucoma, a disease characterized by ganglion cell death. Ideally, cell replacement therapy would be sight-restoring even in late stages of the degenerative process. To improve the efficacy of sight-restoring therapy, the National Eye Institute has established the Audacious Goals Initiative (https://nei.nih.gov/audacious). The purpose of this initiative is to regenerate neurons and neural connections in the eye and visual system. Photoreceptor cells and retinal ganglion cells have been selected as the two compelling target cell types. Relatively few functional cones may be needed to sustain visual acuity of 20/30, which is good enough to support reading and even driving (provided that the peripheral visual field is large enough) [1]. Thus, we probably do not have to have a perfect transplant procedure to bring about a visually important benefit to patients. As has been noted elsewhere [2], the eye has some unique advantages as a target organ for central nervous system (CNS) cell-based therapy in the central nervous system. These advantages are discussed below.

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Advantages of the Eye as a Target Organ

Anatomy and Physiology

The anatomy and physiology of the retina have been characterized in detail (Fig. 1.1) [3, 4]. The neural retina has a laminar organization with PRs abutting against the retinal pigment epithelium (RPE). RPE cells phagocytose the shed PR outer segments (the primary locus of photon capture), provide vitamin A metabolites to the PRs, and contribute to regulation of the intercellular matrix between the PRs and RPE. Cone PRs, which are important for color vision and high-acuity vision, are concentrated in the fovea, which is a pit-like structure in the central retina. Rod PRs support vision in low light environments and are distributed more diffusely throughout the retina. Although integration of transplanted PRs with host retina is complex [5], integration of transplanted RPE with host PRs is, in principle, straightforward

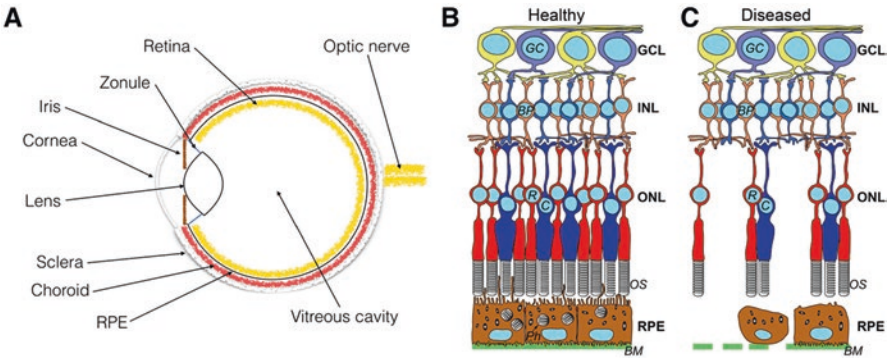

Fig. 1.1 Retinal anatomy in health and disease. (**a**) Diagrammatic representation of the eye. Light passes through the cornea, lens (held in position behind the iris by the zonule), and vitreous gel before encountering the retina. The retinal pigment epithelium (RPE) is interposed between the retina and the subjacent choroid, a vascular layer that provides nourishment to the RPE and the retinal photoreceptors. The sclera, a collagenous outermost layer of the globe, protects the delicate internal structures. The ganglion cell axons comprise the optic nerve, which connects the ganglion cells to the thalamus (lateral geniculate body), the hypothalamus, and midbrain (not shown). (**b**, **c**) Diagrammatic comparison of healthy (**b**) and diseased retina (**c**) showing the dependence of photoreceptors on their support tissue, the RPE. In the diseased retina, such as retinitis pigmentosa, there is loss of rods (R) and cones (C), which eventually is accompanied by a reduction in the number of RPE cells. Additionally, in some diseases, such as age-related macular degeneration, the RPE cells may not be a continuous monolayer and may not be well bound to their substratum, Bruch's membrane (BM). There is reduced phagocytosis of the photoreceptor outer segment (OS), shown as a lack of phagosomes (Ph) in the RPE. Of note, the downstream neural circuitry of the bipolar cells (BP) and the ganglion cells (GC) is depicted as intact although synaptic remodeling often accompanies moderate-severe states of photoreceptor degeneration. *ONL* outer nuclear layer, *INL* inner nuclear layer, *GCL* ganglion cell layer. (**b** and **c**) Reproduced from Ramsden et al. [61] with permission from Karger publishers. (**a**) Reproduced from Zarbin [2] with permission from Elsevier publishers

and occurs spontaneously [6]. In some diseases (e.g., age-related macular degeneration (AMD)), however, transplanted RPE may not establish a healthy monolayer on Bruch's membrane, the collagenous surface on which they reside in situ [7, 8]. Strategies such as cell delivery on scaffolds (see below) or biochemical stimulation of transplanted cells [9, 10] may mitigate these obstacles. The retinal ganglion cells (of which there are 20–30 different types) export processed information about a visual stimulus (e.g., direction, orientation, contrast, looming) to ~46 different structures (e.g., the lateral geniculate nucleus (LGN), the habenula, the amygdala, the superior colliculus) via the optic nerve [11]. The path length (i.e., the distance that axons must extend from the retinal ganglion cell body to their neuronal targets) from the retina to the LGN is ~50 mm. These features render retinal ganglion cell replacement therapy a more challenging enterprise than PR or RPE replacement [12]. Therefore, this chapter focuses on the eye as a target for RPE and PR transplantation.

Surgical Access

Surgical techniques to access to the vitreous cavity and subretinal space are well established and have an excellent safety record. Delivery of cells to the vitreous cavity via transscleral injection using a small-bore needle is a straightforward surgical procedure that can be effective for rescue therapy [13–16]. In some cases these cells exhibit homing behavior and migrate to areas of retinal injury [17, 18].

There is a potential space between the PRs and RPE that can be expanded surgically by injecting fluid through the retina (via a very thin [ranging from 33 to 41-gauge] cannula) into the subretinal space. This maneuver creates surgical access to the subretinal space for the delivery of PRs, RPE cells, and other therapeutic cells [19]. Delivery of cells can be accomplished using a cell suspension or using a scaffold (Fig. 1.2). Subretinal delivery of cell suspensions is relatively straightforward technically and can be accomplished via a small (e.g., 33-gauge) retinotomy, but transplants of RPE suspensions usually do not form a polarized monolayer with uniform orientation of the apical surface towards the PRs, which is essential for proper integration with the PR outer segments. A relatively larger retinotomy is required for delivery of scaffold-adherent cells, which may increase the risk of cell egress into the vitreous cavity and complications such as epiretinal membrane formation and retinal detachment [20]. Scaffolds can migrate in the subretinal space to an extrafoveal location after delivery to a subfoveal locus with possible diminishment of the trophic effects of the transplant and complete loss of a replacement benefit. Some intraoperative techniques (e.g., use of heavier than water liquid such as perfluorodecalin to reattach the retina over the scaffold intraoperatively), however, may mitigate this risk. Use of scaffolds permits one to transplant cells that are differentiated and properly organized anatomically [21], which, in the case of RPE transplants, may reduce PR degeneration during the postoperative period. It also may be possible to integrate survival factors (e.g., neurotrophic factors, immuno-

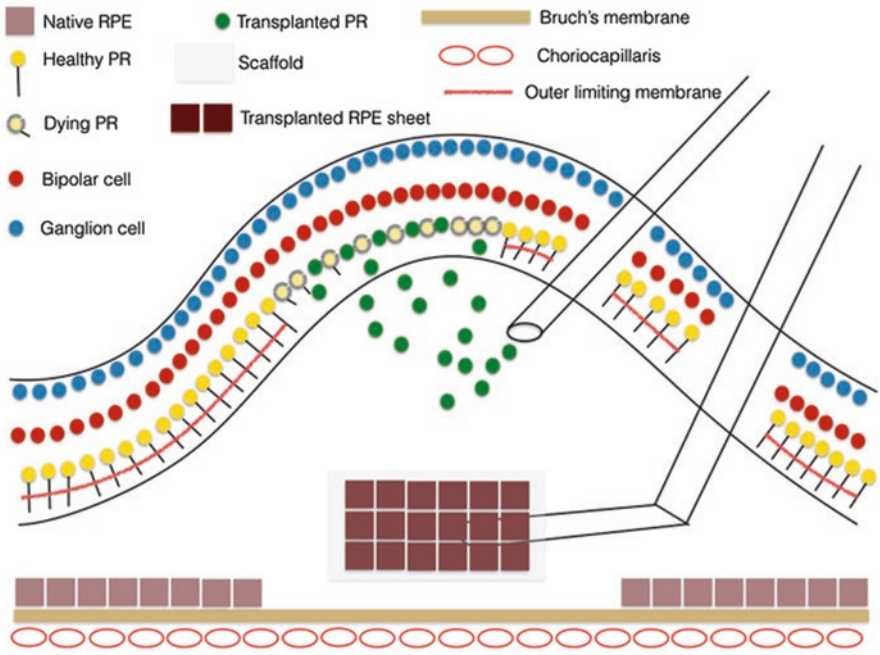

Fig. 1.2 Surgical approach to photoreceptor transplantation. Schematic drawing illustrating subretinal injection of a suspension of rod photoreceptor precursor cells as might be done for a patient with photoreceptor degeneration due to a retinal dystrophy. The cells integrate into the retina preferentially in areas of external limiting membrane breakdown. Also shown is subretinal delivery of an RPE sheet on a scaffold to replace a localized RPE defect on Bruch's membrane as could occur in patients with geographic atrophy. Reproduced from Zarbin [2] with permission from Elsevier publishers

modulatory molecules) into the scaffold to promote transplant and host retina survival. In addition to possibly reducing antigen load (vs. cell suspensions), scaffold delivery systems seem to be associated with better RPE transplant survival and resistance to oxidative damage [22, 23].

Immune Privilege

Under normal circumstances (which may not persist in a diseased state) the subretinal space is an immune privileged site although that privilege is relative rather than absolute [24]. Immune privilege of the subretinal space depends, at least in part, on an intact RPE monolayer [25]. Furthermore, the RPE can modulate an immune response [26, 27]. RPE cells express Fas ligand [28], for example, a transmembrane protein that induces apoptosis in cells expressing the Fas receptor, and Fas ligand/receptor interactions are important in regulation of the immune system.

RPE immune privilege is not absolute [29], and suppression of class II major histocompatibility (MHC) antigens (expression of which can be induced by inflammation) may be necessary for long-term RPE allograft survival [30]. Subretinal allogeneic fetal retina-RPE transplants did not seem to be rejected in humans with RP and advanced AMD in one study [31], but in another, allogeneic subretinal RPE transplants in AMD patients undergoing CNV excision were rejected once immune suppression therapy was stopped [32]. Initial experience with elderly patients receiving RPE transplants indicates that they cannot tolerate triple immune suppression paradigms (i.e., prednisone, cyclosporine, azathioprine) for an extended period of time [32]. Subsequent studies have employed pretreatment and limited posttransplant treatment with mycophenolate mofetil and tacrolimus, but not all patients could tolerate this regimen either [33, 34].

PRs have low MHC class I expression and express MHC class 1b antigens (HLA-G and HLA-E) that bind CD94-NKG2, a lectin receptor expressed on the surface of natural killer (NK) cells, and block NK cell-mediated lysis. Nonetheless, evidence from preclinical models indicates that long-term survival of transplanted PRs requires immune suppression therapy [35]. It is not clear, though, whether this immune response is stimulated solely by the transplanted PRs or whether cellular contaminants might also play a role.

Differentiated progeny of embryonic stem cells (ESC)s express MHC class I antigens [36, 37]. Stem cells generated by somatic cell nuclear transfer are syngeneic to the nuclear donor except for the mitochondrial genes, which are of oocyte origin and are a source of minor histocompatibility antigens [38, 39]. Unfortunately, disparities at the minor histocompatibility loci alone can provoke rejection of ESC-derived tissue [40]. Although induced pluripotent stem cells (iPSCs) might be devoid of alloreactivity, if the iPSC harbors a genetic abnormality and if this abnormality is corrected before transplantation into the iPSC donor, then an immune response may occur [41].

Several strategies are being explored to mitigate the issue of immune rejection. Donor iPSC banks are being created and are targeted for individuals homozygous at some of the MHC loci [42–46]. Potential pitfalls with this approach include the fact that disparities at minor histocompatibility loci can provoke immune rejection, so it is not clear that this approach will be useful for many patients, as well as the fact that although MHC matching could be supplemented with immune suppressive therapy, this approach might be accompanied by an increased risk of ESC-derived tumor formation. Another strategy is to induce tolerance (i.e., absence of a destructive immune response to transplanted tissue without immune suppression) [47]. Tolerance can be achieved via mixed chimerism or by inducing T-lymphocyte anergy through blockade of co-stimulatory signals that activate T cells (e.g., with belatacept [48, 49], a B7-specific fusion protein that inhibits interaction of CD28 with CD80 and CD86). Another approach may be to induce ignorance (i.e., failure of the immune system to recognize transplanted tissue) by exploiting biochemical features of PRs and RPE cells that may suppress an immune response to allogeneic transplants [47].

It is possible that the combination of the immune privileged locus of the transplant (i.e., the subretinal space) and the immune privileged tissue that is transplanted will result in the induction of ignorance. As noted above, PRs have low MHC class I expression and express MHC class 1b antigens (HLA-G and HLA-E) that bind CD94-NKG2 and block NK cell-mediated lysis [47], and RPE cells express Fas ligand [28].

Activation of an inflammatory response can initiate a cascade of events that triggers immune surveillance of transplanted tissue. Minimizing surgical trauma to minimize activation of the innate immune system (e.g., complement proteins, natural killer cells, dendritic cells), which in turn can activate the adaptive immune system (e.g., T- and B-cells), may help to preserve the immune suppressive environment of the subretinal space [50]. Also, one should minimize the use of materials (e.g., cell scaffold components) that induce inflammation and activation of the innate immune system.

Antigen Load

The surface area of the foveal avascular zone is approximately 0.8 mm^2. As a result, the number of cells needed to restore central (high acuity) vision is, in principle, relatively small, e.g., fewer than 1,000,000 PRs and probably fewer than 250,000 [1]. Thus, the antigenic load required to induce a therapeutic benefit should be relatively low, which may aid in reducing the likelihood of immune rejection of allogeneic tissue. Because the immune privilege of the subretinal space may not be absolute [24], particularly in a diseased eye, there may be an advantage to reducing the antigen load with regard to stimulating immune surveillance of the transplanted cells [51]. The use of scaffolds for cell delivery may have several potential advantages [22, 23], one of which is reducing the number of cells that need to be transplanted in order to restore visual function.

High-Resolution Noninvasive Imaging Technology

Ocular tissue can be imaged in the living human eye with remarkably high resolution using noninvasive technologies [52–54]. This issue is explored in great detail elsewhere in this book, so some technologies will only be mentioned briefly here. Optical coherence tomography (OCT), for example, provides 3 μm resolution transverse images of the retina (rivaling histological specimens in resolution), and scanning laser ophthalmoscopy adaptive optics imaging permits one to visualize individual PRs within a mosaic over an extended period of time [55]. Individual RPE cells also can be visualized using adaptive optics scanning laser ophthalmoscopy [56]. In addition, techniques such as fluorescein angiography permit detailed assessment of some aspects of ocular physiology such as the integrity of the

blood-ocular barrier. One can visualize retinal and choroidal blood vessels using noninvasive imaging techniques such as OCT angiography [57–59]. As a result, one can monitor closely the transplanted tissue's anatomy as well as the anatomy of the recipient retina in a patient after surgery.

Functional Assessment of the Transplant

Electrodiagnostic testing, such as the electroretinogram and the multifocal electroretinogram, and psychophysical testing, such as microperimetry, allow one to assess functional recovery in ways that are more incremental than the gross psychophysical measurement of visual acuity. Gene therapy experiments in the $Gnat^{1-/-}$ mouse model of congenital stationary night blindness indicate that approximately 150,000 functioning rods are required to generate a reproducible dark-adapted (scotopic) ERG signal [60]. (Restored visual *behavior*, however, can be detected with only 25,000 functioning PRs in this model.) Since the electroretinogram is essentially non-recordable in advanced RP patients, functionality of rod PR transplants might be monitored using this technology. These imaging and monitoring capabilities permit developing an iterative pathway to successful transplant paradigms in human patients as well as modulating immunotherapy precisely should it be needed.

Conclusions

The eye is well suited for initial studies of CNS cell-based therapy due to: the well-defined anatomy and physiology of the retina and supporting tissues; the existence of safe effective surgical techniques for cell delivery; the relative immune privilege of the eye and, in particular, the subretinal space; the likely need for relatively few viable transplanted cells to support site restoration; the availability of noninvasive imaging technology to monitor serially the transplant and host anatomy at the micron scale; and the ability to assess transplant functionality incrementally using noninvasive electrodiagnostic and psychophysical tests. There are a large number of patients with currently untreatable forms of blindness and for whom cell-based therapy holds great promise. These facts, taken together with the success of cell-based therapy in preclinical models of retinal degenerative disease, suggest that sight-preservation/restoration will be among the earliest examples of successful cell-based therapy in the CNS.

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Chapter 2
Proof of Principle: Preclinical Data on Retinal Cell Transplantation

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Introduction

Decades of preclinical research to develop cell transplantation treatments for retinal degenerative diseases have now culminated in human clinical trials. Retinal degenerative diseases cause vision loss through the dysfunction and/or degeneration of retinal photoreceptor cells, and so cell-based treatments for these conditions aim either to rescue photoreceptor cells from continued degeneration or to regenerate functional photoreceptor cells. In the former strategy, the introduction of cells into or near the retina creates a rescue effect though the release of soluble factors that promote photoreceptor survival and function. Transplantable cell types for this purpose include retinal pigment epithelium (RPE) cells, neural and retinal progenitor cells, bone marrow cells, and umbilical tissue-derived cells. In the latter strategy, cells are introduced (into the subretinal space) that have the capacity to mature into functioning photoreceptor cells so that visual function can be restored. This chapter aims to review and summarize preclinical data on retinal cell transplantation in the context of the proposed mode of action of rescuing or regenerating retinal photoreceptor cells in retinal degenerative diseases.

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Photoreceptor Rescue Therapy

RPE Transplantation

The RPE is a monolayer of cuboidal pigmented cells that normally lies adjacent to the photoreceptor layer. RPE cells are not neurons, and so are not considered a part of the visual pathway, but instead provide support to photoreceptor cells. Microvilli on the apical surface of RPE cells interact closely with photoreceptor outer segments [1]. Through this interaction, RPE cells provide trophic, metabolic, and other forms of support for photoreceptor cells [2, 3]. RPE cells recycle vitamin A derivatives, which are a critical component of the visual cycle. In order for photoreceptors to function, they must receive a constant supply of 11-cis retinal from RPE cells [4–8]. A review on key physiological actions of RPE cells in relation to photoreceptor maintenance is beyond the scope of this chapter; however, the reader is referred to a detailed review on this subject by Bok [4].

Because photoreceptors and RPE cells are physiological partners in the retina, the therapeutic effect of RPE transplantation lies in conferring functional benefit on the physiological status photoreceptor cells. RPE transplantation has been shown to preserve visual function—i.e., slow down or halt the process of photoreceptor degeneration—when transplanted into recipients with early-stage or partial retinal degeneration, when sufficient photoreceptor cells remain that are amenable to rescue. Extensive data from murine models of human retinal degeneration, including the Royal College of Surgeons (RCS) rat and *Rpe65*$^{-/-}$ mouse, support this treatment concept [9–17]. Specifically, rescued photoreceptors in the vicinity of the RPE transplants appear to show normal metabolism and rod outer segment renewal rate [13], indicating a local paracrine effect that sustains or improves photoreceptor cells nearby. It must be noted, however, that transplantation outcome data using the RCS rat model should be interpreted with caution, because photoreceptor rescue effects can be detected for up to 2 months in this model from temporary surgical retinal detachment alone, without the delivery of cells or other therapeutic agents [18].

RPE cells normally rest on Bruch membrane, and the latter is known to display pathological changes in patients with retinal degenerative diseases. Extensive work has been done to investigate the influence of Bruch membrane on the health and survival of RPE in culture and following transplantation. Dissociated human fetal RPE cells spread over Bruch membrane, develop a hexagonal shape, form tight junctions, and develop apical microvilli over a period of approximately 24 h [19]. Aged human Bruch membrane is an imperfect substrate for the efficient attachment and stabilization of transplanted RPE cells [20], and so surface modification of aged Bruch membrane (for example, with exogenous cell matrix [21]) will likely be beneficial in the diseased human recipient. Efforts have also been made to design synthetic replacements for Bruch membrane. Such a replacement should have permeability characteristics that approximate native Bruch membrane in order to allow for the diffusion of materials between the choroid and photoreceptor cells. Parylene C of 0.15–0.30 μm thickness exhibits permeability that is similar to healthy

Bruch membrane, and also supports the adherence and proliferation of RPE cells in culture [22]. Other materials with favorable characteristics for this purpose include collagen [23], poly(DL-lactic-co-glycolic acid) (PLGA) [24, 25], poly(L-lactic acid) (PLLA)–PLGA blends [26], elastin-like recombinamers [27], polyimide [28], biodegradable polyurethanes [29], Descemet membrane [30], amniotic membrane [31], poly(e-caprolactone) [32], poly(glycerol-sebacate) [33], and polyester [34].

The use of ex vivo scaffolds also supports the goal of developing preformed sheets of RPE cells prior to transplantation because the RPE cells can be cultured in vitro on the scaffold prior to transplantation. In this paradigm, the surface provided for the attachment and proliferation of the donor RPE cells can be optimized [21], and the donor cells remain on that same surface after transplantation. Hence, the potential negative influence of the aged or diseased recipient Bruch membrane on the transplanted RPE cells [35] may be mitigated.

Murine models are usually inadequate for the study of preformed sheet retinal cell transplantation owing to their small size. Typically, small-bore needles are used for transplantation, and these are generally unsuitable for the delivery of RPE cell sheets which are larger in size and more susceptible to structural damage if passed through small-bore needles. Therefore, larger animal models such as the pig and rabbit [36, 37] have been useful to investigate the transplantation of RPE sheets [34, 38]. Using the pig model, subretinal allogeneic RPE sheet grafts have been shown to survive for up to 3 months [39]. The rabbit model was used in a study of subretinal transplantation of adult human RPE stem cells grown on polyester membranes. Four weeks after transplantation, a stable polarized human RPE monolayer on a polyethylene terephthalate (PET) membrane was detected histologically (Fig. 2.1) [34]. Successful sheet delivery using vitrectomy techniques was demonstrated in the pig model using primary porcine

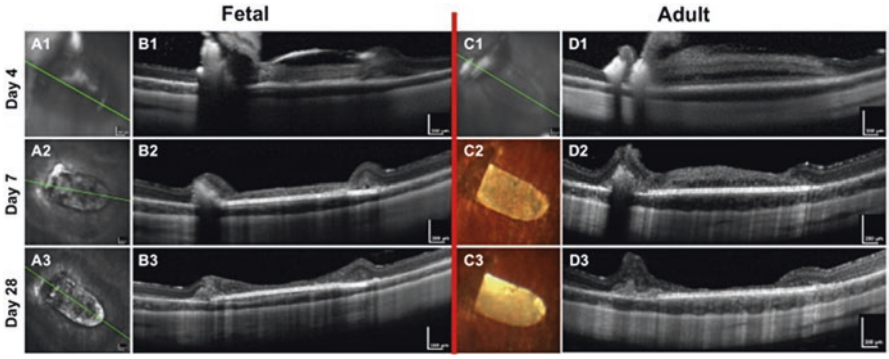

Fig. 2.1 Fetal and adult human retinal pigment epithelium on polyester membranes transplanted into the rabbit subretinal space, showing intact transplanted constructs up to 28 days after the procedure. (**A** and **C**) Fundoscopy images, (*A1–A3* and *C1*) infrared confocal scanning laser ophthalmoscopy images, (*C2* and *C3*) color fundus photographs, (*A4* and *C4*) postmortem macroscopic photographs. (**B** and **D**) Longitudinal optical coherence tomography section through the center of the implants at indicated time points. (*B1–B3* and *D1–D3*) Spectral domain optical coherence tomography images. Scale bars, 200 µm. (Figure and legend extracted and adapted from Stanzel, B.V., et al., Stem Cell Reports, 2014. 2(1): p. 64–77) [34] (CC-BY licence)

RPE sheets as the donor substrate [39]. However, a recent study showed that smaller models can be used in some cases. Using a rat model, investigators successfully demonstrated engraftment of human embryonic stem cell (hESC)-derived RPE cell sheets grown on a human amniotic membrane scaffold with effective rescue of photoreceptor degeneration. This study also demonstrated the superiority of the tissue-engineered RPE sheet approach compared to the same cells injected in the form of a bolus of dissociated cells in suspension [31]. A new delivery tool was recently developed for large animal transplantation through a 1.5 mm sclerotomy and a 1.0–1.5 mm retinectomy. This tool is a custom 17-gauge tissue injector and will likely enable the delivery of cellular sheets of considerable size. The scaffold holding the cells is reported as being able to curl inside the tube without overlapping the edges, preventing cell loss. The injector folds the substrate, decreasing the size of the sclerotomy. The authors concluded that their novel device minimized tissue trauma and postoperative inflammation [40].

Interestingly, recent data suggest that the developmental stage of stem cell-derived RPE may be an important factor determining the efficacy of RPE cell replacement. In a study using RPE stem cell derived RPE, cells that were differentiated for 4 weeks of culture had a more consistent functional impact than cell progeny that were differentiated for shorter (2 weeks) or longer (8 weeks) in vitro prior to transplantation [41]. The concept of an ontogenetic window that defines donor cell competence is well known to apply to rod photoreceptor cells, whereby only donor cells harvested during a narrow window of cell maturity, related to specific developmental milestones, can integrate into the recipient retina [42] and restore vision [43]. It was thought that this age window defined the capacity of donor photoreceptors to form synapses with the recipient. So, it is surprising that a similar concept applies to RPE cells which do not form synapses in the retina.

Neural and Retinal Progenitor Cell Transplantation

Among the first data to delineate the potential of using neural progenitor cells as a means to rescue retinal cells from degeneration in the diseased adult retina came from Young et al. in 2000, where they transplanted green fluorescent protein (GFP)-expressing adult rat hippocampal progenitor cells into retinal degenerate rats and were able to subsequently observe neuronal differentiation and morphological integration of the progenitor cells in the dystrophic retina of the recipient [44]. Their data supported the idea that neural progenitor cells could respond to injury or degeneration cues in the retina. In other work, isolated populations of murine retinal progenitor cells were obtained by extracting the retinas of postnatal day-1 mice and removing the ciliary marginal zone. Cultured cells were transplanted into the degenerated retinae of two retinal degenerate mouse strains—C57BL/6 *rho*$^{-/-}$ mice or C3H *rd* mice—wherein they showed signs of integration, maturation into photoreceptors, and rescue of cells in the outer nuclear layer [45].

Data from large animal studies indicate that retinal progenitor cells not only confer a rescue effect but could also potentially regenerate photoreceptor cells. Klassen et al. showed that cultured porcine retinal progenitor cells survived for up

to 5 weeks without immunosuppression in a pig model [45]. To create the cell culture, they collected eyes from fetal pigs at 60 days gestational age. The neural retina was then removed, minced, and enzymatically digested to yield cells which were cultured with additional growth factors. After transplantation, immunohistochemical analysis showed that survival of the donor cells was substantial at 1–2 weeks but variable at 5 weeks. In order to track donor cells more effectively, similar transplant procedures were conducted using GFP transgenic pigs as the donor. Immunohistochemical (IHC) data using this donor model revealed that the donor cells survived up to 10 weeks without immunosuppression after transplantation into allorecipients. Some of the cells integrated into the retina and formed rosettes, indicating possible photoreceptor differentiation [46].

In contrast to allogeneic transplantations, Warfvinge explored xeno-transplantation of progenitor cells into the subretinal space of the pig. Without the use of immunosuppression, transplantation of murine and human progenitor cells to the subretinal space of pigs led to weak integration and survival of the grafted cells [38, 47, 48]—suggesting that modulation of recipient immunity is probably required to ensure xenograft survival in preclinical studies. However, the relative immune privilege of the subretinal space may ameliorate the immune reaction against xenografts to some degree, as data using light-induced retinal degenerate minipigs as recipients demonstrated functional retinal improvements after transplantation of human fetal neuroretina and RPE with no mention of immunosuppression [49].

Bone Marrow Cell Transplantation

Interest in using bone marrow stem cells (BMSC) as a therapeutic substrate for retinal rescue and repair arose from early observations that BMSC could differentiate into various mature adult cell types [50–53], including neurons [54, 55], astrocytes [56, 57], and retinal neural cells [58]. BMSC are an attractive substrate because they are a readily available source of proliferating cells for autologous transplantation, unlike retinal or brain cells. Tomita et al. induced retinal injury and intravitreally injected what was considered to be the stem cell-enriched fraction of low-density bone marrow cells (BMCs). After 2 weeks, the injected cells expressed antigens that were specific to retinal nerve cells, including rhodopsin, indicating possible transdifferentiation into retinal neurons in response to local cues in the injured retina [58]. It appears possible to convert bone marrow cells into photoreceptor-like cells in vitro, prior to transplantation. Hence, pre-differentiating bone marrow cells into retinal neurons could be one strategy to increase the effectiveness of this treatment strategy. Adult CD90+ bone marrow stromal cells can be partially induced by activin A, taurine, and EGF into cells that express photoreceptor-specific markers. These partially differentiated retinal cells, when transplanted into adult RCS rats, underwent differentiation to form structures that bore resemblance to the photoreceptor layer [59]. Retinal transdifferentiation of bone marrow cells has also been seen when the latter were injected into the light injury mouse model [60].

In terms of a rescue effect—in contradistinction to a retinal regeneration effect—bone marrow cells are known to ameliorate neuronal and retinal degeneration [60–63]. Unselected murine bone marrow cells, deposited subretinally in the RCS rat, resulted in the relative preservation of photoreceptor cell bodies and electrophysiological retinal function for up to 8 weeks following the procedure [64]. A similar effect has also been noted following bone marrow cell transplantation in the rhodopsin knockout mouse [65]. Significant retinal neuroprotection can also be achieved when bone marrow cells are injected into a peripheral vein instead of being delivered directly into the eye [66], and so perhaps ocular surgery can be avoided in future human applications of this treatment approach. Mechanisms of neuroprotection that have been proposed include a paracrine effect involving molecules secreted directly from bone marrow cells [64], or possibly following their transdifferentiation of the bone marrow cells into RPE cells following transplantation [65]. The complete set of molecular mediators of retinal neuroprotection by bone marrow cells is unknown, but could include BDNF [60, 67] and other molecules with similar actions. Recent observations have revealed an additional mechanism underlying the therapeutic effect of bone marrow-derived cells. In experiments using the *rd10* retinal degeneration mouse model, transplanted hematopoietic stem and progenitor cells fused with Müller glia and reprogrammed the latter into intermediate photoreceptor precursors that differentiated into mature photoreceptors in situ [68].

Adult bone marrow contains a specific population of endothelial precursor cells (EPCs) that are capable of forming blood vessels. EPCs interact with retinal astrocytes, and when injected intravitreally, rescue the vascular degenerative process that occurs in the *rd* mouse model [69]. Hence, retinal diseases which feature a reduction in retinal vascular density or flow may benefit from intraocular administration of EPCs. It should be noted that photoreceptor and RPE cells are supplied by the choroidal circulation, and not the retinal blood vessels. Based on available data, it is unclear how bone marrow cell transplantation could be used to increase choroidal blood flow or choroidal vessel density. The precise mechanistic link between vascular and neuronal degeneration in the retina is unclear, but interestingly EPC transplantation has been shown to confer a neurotrophic rescue effect in mouse retinal degeneration models, possibly through an antiapoptotic mechanism, in concert with its vascular rescue effect [70].

Umbilical Tissue-Derived Cell Transplantation

Early data comparing the photoreceptor rescue effect of human umbilical tissue-derived cells (hUTCs), placenta-derived cells, and mesenchymal stem cells indicated that umbilical cells provided the strongest rescue effect in the RCS rat model when delivered intravitreally [71]. hUTCs originate from extraembryonic mesoderm, are capable of significant expansion [71], and are not considered to be actual stem cells because they are not known to differentiate into other cell types.

Umbilical tissue-derived cells are thought to rescue phagocytic dysfunction in RCS RPE cells by secreting brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), and glial cell-derived neurotrophic factor (GDNF) and other molecules including bridge molecules that structurally facilitate ingestion of photoreceptor outer segments by the RPE [72]. Excitingly, hUTCs have been shown to promote synaptogenesis and support neuronal growth, and these effects are mediated by thrombospondins [73]. hUTC transplantation is under investigation in a Phase I human clinical trial (NCT00458575), and currently available results indicate that the cell product is well tolerated when placed in the subretinal space and may be associated with improved visual function in human subjects [74].

Photoreceptor Replacement Therapy

Photoreceptor cells are the cornerstone of vision, being the primary cells in the mammalian retina that respond directly to light stimulation. The human retina contains four types of photoreceptors located in the outer retina which contribute to visual functions, namely rod photoreceptor cells, and short,- medium-, and long-wavelength sensitive cone photoreceptor cells. One additional class of nonvisual photoreceptor—the intrinsically photosensitive retinal ganglion cell—is located in the inner retina, and functions mainly to regulate circadian rhythms [75]. The sections that follow will focus on the replacement and regeneration of the rod and cone photoreceptor cells.

Neuroretinal Sheet Transplantation

The strategy of neuroretinal sheet transplantation involves the harvest and transplantation of intact sheets of retina, in contrast to the other strategy discussed below in which retinal sheets are enzymatically digested to yield dissociated photoreceptor cells in suspension. Primary tissue refers to that which is harvested from a living or cadaveric donor. Transplanting retinal tissue as preformed sheets—therefore maintaining their in situ cellular organization—is thought to have a number of advantages including greater transplanted cell survival, more efficient maturation, and more physiological graft architecture. In fact, mature photoreceptor outer segments are known to form more efficiently when neural retina is transplanted without prior enzymatic dissociation into single cells [76]. However, depending on the protocol for tissue harvest and preparation, neuroretinal sheets typically contain large numbers of non-photoreceptor cells which could pose a barrier to the integration of transplanted photoreceptor cells with recipient bipolar cells. In addition, transplanted glia might activate immune surveillance of the transplant. Primary neuroretinal transplants are not likely to be a viable clinical strategy because cadaveric retina degenerates very soon after death, and retinal tissue cannot easily be obtained from living donors without significant surgical morbidity. However,

the investigation of primary neuroretinal transplantation has been useful to better understand the biology of retinal regeneration and to develop useful insights that can be applied to stem cell-based strategies. Effective stem cell-based strategies to create large sheets of preformed retina in vivo have not yet been developed, although relatively small sheets up to about 4 mm in diameter can be generated in stem cell-derived retinal organoids that are discussed below. The size limitation of the organoids produced from current protocols is probably due to the absence of a vascular supply in vitro.

Among the first experiments to show the effects of transplanting sheets of primary rat retina (from an embryonic donor into a mature recipient with induced traumatic retinal lesions) were performed by Turner and Blair. In their experiments they showed that embryonic retinal grafts could survive and potentially integrate in the lesion site, effectively bridging the retinal area that was wounded with a surgical incision [77]. The use of primary sheets of retina as a transplant substrate was further investigated by Aramant and Seiler, who showed effective methods of transplanting sheets of retinal tissue which could survive in the recipient retina [78–80]. Unfortunately, there was a lack of robust integration between donor and recipient retina. A low efficiency of synaptic integration of the full thickness sheet with recipient neurons was shown using lentiviral labeling of the donor retina [81]. In studies of retinal sheet transplantation in pigs, the grafted retina, in most cases, formed a laminated sheet that could be distinguished from the recipient retina. However, similar to the murine experimental results, the graft and recipient retina did not integrate closely with one another [82]. Subsequent data using a rhodopsin transgenic pig model as the recipient showed a lack of axonal processes growing from the grafted retina into the recipient retina [83, 84]. Full thickness retina sheets that were placed in culture for transplantation remained stable for 6 months after transplantation into a rhodopsin transgenic pig but with minimal integration—nonetheless, the cone-mediated retinal responses showed an apparent improvement in treated eyes, possibly due to a synapse-independent mechanism [85]. The data indicate that while transplanted retinal sheets can survive and stably maintain their planar orientation in the subretinal space, further work is required to improve synapse formation between transplanted and recipient tissue.

Photoreceptor rosettes are an abnormal anatomical feature, wherein photoreceptor orientation and alignment are disrupted, and the photoreceptors lose their normal spatial relationship with RPE cells—hence, extensive rosette formation is a negative outcome of retinal transplantation. Aramant and Seiler proposed one hypothetical cause, that small tears along the edges of micro-sheets of donor neuroretina were responsible for the formation of rosette structures, where the retina folds up on itself, preventing correct integration of the neurons into the recipient [78]. To avoid this phenomenon, they prepared their micro-sheets of retina for transplantation by encapsulating them in matrigel for protection. Furthermore, they designed a custom surgical tool for transplantation which would "place" the sheets, rather than injecting them, thus reducing rosette formation [80]. These important data show that the mechanical preparation and surgical delivery of cellular retinal sheets are important determinants of the outcome of retinal transplantation. As rosette formation continues to be a feature in more recent studies using ESC and iPSC derivatives [86], encapsulation strategies could be applied so that this outcome can be avoided.

The apparent lack of integration of donor retinae with recipient tissue could also be due to a barrier created by gliosis, which is precipitated by the upregulation of glial filament intermediate proteins (GFAP) and vimentin in the recipient Müller cells [87]. By transplanting neuroretina into mice deficient in GFAP and vimentin, robust integration of the donor neuroretina into the CNS of the recipient can be achieved, including correct neuronal identity and projections [88]. Disruption of the glial seal by enzymatic means through chondroitinase ABC (ChABC) treatment has also been shown to promote synapse formation between graft and recipient neurons following retinal transplantation [89].

As mentioned above, the continued health and survival of photoreceptors in vivo—and therefore presumably of transplanted exogenous photoreceptor cells—depends on their interaction with viable RPE cells. In many retinal degenerative diseases, RPE cell degeneration occurs along with neural retinal degeneration, and hence the total cellular depletion in the recipient involves both photoreceptors and RPE cells. If photoreceptor cells alone were transplanted during a treatment procedure, they would likely not survive into the long-term, nor function optimally, as they would lack the trophic support of RPE cells. The transplantation of both cell types has been shown to be required for correct lamination of the neural retina [79]. RPE also has a role in determining photoreceptor arrangement, acting through diffusible factors on immature Möller glial cells to co-ordinate the arrangement of photoreceptors [90]. Thus, it would be advantageous to augment the transplantation of photoreceptor cells with RPE cells, although significant work remains to be done in this area of optimization.

Photoreceptor Cell Transplantation

Techniques were developed to isolate rod and cone photoreceptor cells for transplantation for a number of reasons. By enzymatically dissociating harvested primary retina—typically with enzymes such as papain or trypsin—a mix of individual retinal cells is obtained, which can then be further enriched for rod and cone photoreceptors by using magnetic- or fluorescence-based selection strategies. Transplanting these purified rod and/or cone photoreceptor cells would enable the more targeted study of the biology underlying their maturation and integration following transplantation. Also, the potentially negative influence of non-photoreceptor cell types—for example, the barrier effect posed by donor inner retinal neurons such as ganglion and bipolar cells—is reduced or eliminated, thereby potentially enabling better integration between donor and recipient. Compared with intact sheets, dissociated cell populations suspended in solution are easier to transplant into small animal eyes (e.g., rodent) as a bolus using small gauge needles.

Anatomical and functional preservation of the recipient retina have been shown in numerous murine models of photoreceptor cell transplantation [42, 91–94]. There has been, in general, a low efficiency of cellular survival and integration, and this result may be due to some extent to the limited cells survival of the transplanted cells.

Factors influencing the survival of grafted cells likely include immunological rejection and the physiological stress associated with the cell preparation (e.g., enzyme exposure) and transplantation protocol (e.g., shear forces on the cells during injection through small gauge needles). Antiapoptotic treatment of the donor cells could be used as one strategy to increase transplanted cell survival [93], as can the ectopic expression of neurotrophic growth factors [95]. In the setting of severe retinal degeneration, with near-total loss of retinal photoreceptor cells (using aged *rd1* mice, which typically exhibit a severe and rapid photoreceptor degeneration phenotype, as the recipient), photoreceptor transplantation regenerates photoreceptor cells in the recipient and restores visual responses that were absent prior to treatment [43] when the donor cells were harvested during the narrow ontogenetic window of integration competence that was demonstrated previously [42]. More recent data in pigs have validated this narrow developmental window for the integration and differentiation of primary cone and rod precursors following transplantation, wherein maximal survival and integration of rod and cone photoreceptors was obtained only when they were harvested and transplanted at or around the time of their genesis in the donor [96]. Mouse data indicate, however, that older donor cells, harvested outside this window also retain their ability to survive and integrate to some extent [97].

The positive functional effects have been validated using stem cell-derived donor photoreceptor cells [98]. Human embryonic stem cell-derived retinal precursors, amplified in two-dimensional culture and transplanted into *CRX*-deficient mice, showed evidence of maturation into cone and rod photoreceptors and restored the visual light responses in the animals [99]. Investigators in Japan generated three-dimensional stem cell-derived retinal organoids, first using murine stem cells [100], and then human embryonic stem cells [101]. These three-dimensional organoids recapitulated the growth and development of the eye field in embryonic development, and showed a remarkable ability to self-organize, generating multiple distinct layers of the neuroretina in correct polarization and orientation, including rod and cone photoreceptors. Photoreceptors from these three-dimensional human stem cell-derived organoids are transplantable into mice, showing evidence of recipient-graft synaptic connections and photoreceptor outer segment maturation (Fig. 2.2) [102].

Photoreceptors from these three-dimensional human stem cell-derived organoids have also been investigated in nonhuman primate models [86]. In the first model, a subretinal injection of cobalt chloride induced complete outer nuclear layer (ONL) loss, and other layers of the retina remained intact as demonstrated with immunostaining for amacrine, horizontal, and bipolar cells. The other model utilized 577 nm laser photocoagulation to selectively reduce photoreceptor layer thickness; however, the degeneration of the photoreceptor layer was neither uniform nor complete. Both methods resulted in reduced local function (measured by electroretinographic amplitudes) corresponding to the injury sites. Differentiation-day 60 (DD60) tissue from human three-dimensional stem cell-derived organoids were transplanted into these nonhuman primate models. Sequential in vivo imaging revealed that the grafts increased in size until around DD120 and remained stable thereafter. In both models, the grafts ultimately formed rosettes. The data indicate that stem cell-derived retinal photoreceptor cells are transplantable and show prolonged survival; however, the maturation of

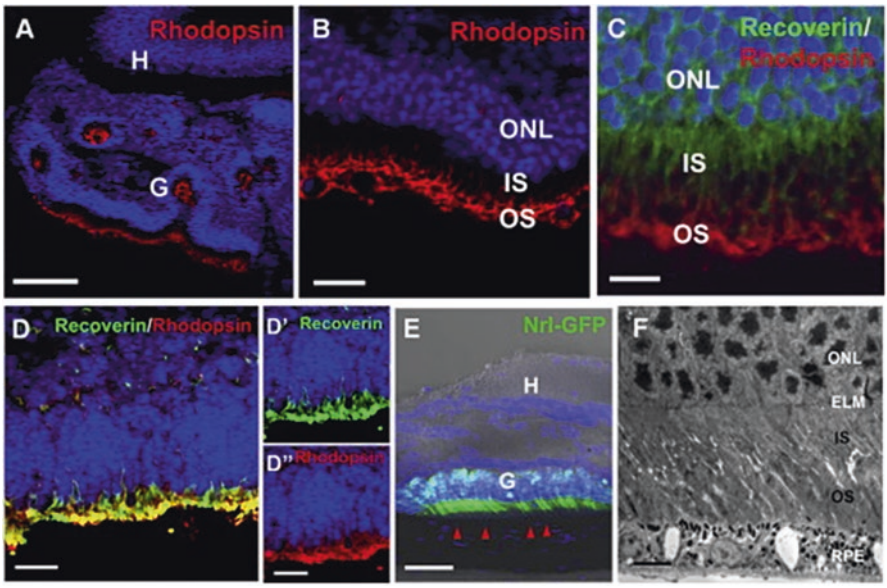

Fig. 2.2 Transplanted retina-like sheets show photoreceptor inner segment/ outer segment (IS/OS) formation. (**A**–**C**) Differentiation day (DD) 14 Nrl-GFP mouse induced pluripotent stem cell (miPSC)-derived retinal graft showed IS/OS. IS was labeled with recoverin; OS was labeled with rhodopsin (**C**). (*D*–*D*″) Rx-GFP mouse embryonic stem cell-derived retinal culture at DD26 showed short IS/OS colabeled with recoverin and rhodopsin. (**E–J**) DD17 Nrl-GFP miPSC-derived retinal graft with IS/OS contact host retinal pigment epithelium (RPE, red arrowhead) more than 4 months posttransplantation. (**F**) Electron microscopy showed that rod nuclei in the outer nuclear layer contained a compact mass of heterochromatin, external limiting membrane, IS/OS, and RPE. Scale bars, 100 μm (**A**), 50 μm (*D*–*D*″ and **E**), 20 μm (**B** and **F**), 10 μm (**C**). *Nrl* neural leucine zipper, *GFP* green fluorescent protein. (Figure and legend extracted and adapted from Assawachananont, J., et al., Stem Cell Reports, 2014. 2(5): p. 662–74) with permission from Cell Press [102]

the graft and the resulting tissue architecture of photoreceptor arrangement is as yet suboptimal. Notably, the grafted hESC photoreceptors demonstrated direct integration with recipient bipolar cells in a number of graft locations in which the graft bipolar cells did not block contact between the graft photoreceptors and recipient bipolar cells [86]. In another study, the injection of hESC cells which had been differentiated towards neuronal fates into squirrel monkey eyes survived at least 3 months post injection without immunosuppression, and the donor cells appeared to integrate into the recipient retina with some also projecting into the optic nerve [103].

These data provide proof of principle that stem cell-derived photoreceptor cells can be used as a therapeutic substrate to regenerate the retina. Further progress has since been made in the generation of more advanced three-dimensional organoids. Modifications of the original protocol have resulted in more efficient differentiation of the cells into photoreceptors with evidence of outer segment disc formation in vitro [104, 105]. Hence, potentially better stem cell-derived therapeutic substrates could soon become available for regenerative transplantation.

The majority of published protocols for generating photoreceptors, whether in two dimensions or three, tend to generate rod photoreceptor-rich retinal tissue. Rod photoreceptors will be useful to treat patients with predominant rod photoreceptor loss; however, patients with macular dystrophies will more likely benefit from cone-rich photoreceptor transplantation. Data on successful differentiation and transplantation of cone photoreceptors have been described [106]. For example, the use of *COCO*, a *Dand5* member of the Cerberus gene family, can generate sheets of photoreceptors with a short wavelength sensitive cone identity [107]. More recent data indicate that treatment of murine embryonic stem cells with the Notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) at days 16–18 of differentiation substantially increased the levels of S-opsin expression in three-dimensional organoids from ~8% to ~16%, suggesting that inhibition of the Notch pathway may be a method of increasing cone photoreceptor yields for transplantation [108]. Increased numbers of cone photoreceptors can also be generated in human stem cell-derived three-dimensional organoid protocols which utilize a combined 2D/3D approach [109].

Retinal Cellular Materials Transfer

In many of the papers mentioned above, the authors transplanted fluorescent cells and used images of this fluorescence in the recipient retina as evidence that the transplanted cells had integrated into the recipient retina. However, it is now known that donor-derived fluorescence in the recipient following transplantation is due to some extent to the transfer of cytoplasmic material from individual donor cells to recipient cells [110–113]. The data indicate that the transfer of materials can occur in a bidirectional manner, i.e., from donor to recipient cells and vice versa [111], and also that nuclear fusion is not likely to be involved [112]. The results of these experiments collectively indicate that the majority of the results seen so far in photoreceptor transplant studies are the result of material transfer into recipient cells and not the integration of donor cells as previously thought. Further experiments will need to be performed to determine the mechanism by which cytoplasmic material transfer is taking place, and the implications this may have on the prospect of using cell transplants for retinal repair.

Conclusion

A vast body of preclinical data on retinal cell transplantation supports the concept of cell-based retinal rescue and regeneration as treatment modalities for retinal degenerative diseases. In patients with mild, early, or localized retinal degeneration, the rescue strategy can be employed, wherein supportive cell types such as RPE or bone marrow cells are transplanted into the eye in order to arrest the degenerative

process. In contrast, in severe retinal degeneration, photoreceptor transplantation is envisioned to provide a means to directly regenerate retinal photoreceptors in order to restore visual function. Recent advances in retinal stem cell technology, including new techniques to generate rod and cone photoreceptor cells in culture through defined stem cell protocols, have provided the means to produce human replacement cells that will not rely on primary donors and so can be scaled up for widespread clinical application. Newly discovered mechanisms such as photoreceptor cytoplasmic transfer may lead to the development of new treatment strategies in the future.

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Chapter 3
Clinical and Pathological Features of Selected Human Retinal Degenerative Diseases

Michael C. Hogden and Stephen Tsang

Introduction

Classification of retinal degenerative diseases has traditionally relied on a phenotypic description based on fundus appearance alone. However, with the advent of more rapid and cost-effective genetic testing, this paradigm has changed, with a new clinical focus on establishing an accurate genetic diagnosis and correlating these findings with the traditional phenotypic description. As well, the phenotypic description itself has expanded to encompass not only the fundus appearance but also relevant investigative findings, including optical coherence tomography (OCT), fundus autofluorescence, retinal angiography, and electrodiagnostics. Ultimately, all of these recent advances have paved the way for more accurate patient diagnosis and facilitated new insights into the potential for individually tailored cell-based therapy.

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Generalized Retinal Degenerations

Retinitis Pigmentosa

Clinical Characteristics

Retinitis pigmentosa (RP) is the term used for a group of inherited retinal degenerative disorders characterized by progressive rod-cone dysfunction and eventual atrophy of both rods and cones throughout the retina. RP may occur in isolation (termed "typical" RP) or may occur in association with systemic disorders (termed "syndromic" RP). The prevalence of typical RP has been documented as approximately 1:5000 worldwide [1–5].

Although disease progression in RP can vary, the two hallmark symptoms of RP include (1) nyctalopia (night blindness) and (2) an insidious, progressive loss of peripheral visual field, due primarily to rod and subsequently to cone degeneration. Central visual dysfunction may also occur, due to a combination of cone degeneration, cystoid macular edema (CME) [6], and/or macula pucker [7].

Important syndromic forms of RP include [8]:

- Frequent syndromes.
  - Usher syndrome.
  - Bardet Biedl syndrome.
- Less frequent syndromes.
  - Renal abnormalities: Senior Loken syndrome, Alport syndrome.
  - Dysmorphic syndromes: Cohen syndrome, Jeune syndrome, Cockayne syndrome.
  - Metabolic diseases: Methylmalonic aciduria with homocystinuria, abetalipoproteinuria (Bassen Kornzweig disease), Bietti's disease, cystinosis, mucopolysaccharidoses (Types I, II and III), Zellweger (cerebro-hepato-renal) syndrome, hyperoxaluria type I, neonatal adrenoleukodystrophy, Refsum disease.
  - Neurological diseases: Neuronal ceroid lipofuscinosis (Batten disease), Joubert syndrome, autosomal dominant cerebellar ataxia type II (SCA7), myotonic dystrophy, Hallervorden-Spatz syndrome.

Genetic Associations and Pathophysiology

Clinicians, cell biologists, and molecular geneticists have not yet developed a unified subclassification system for RP based on an amalgamation of molecular, biochemical, and clinical features. However, the most useful subclassification in both the clinical and research settings may be based on mode of inheritance.

Typical RP can be inherited in an autosomal dominant (adRP), autosomal recessive (arRP), or X-linked recessive pattern (xlRP). Autosomal recessive is the most common and is associated with significant genetic heterogeneity [9]. All typical RP is genetic, but there is a lack of a positive family history in approximately 50% (termed "simple RP"). It is estimated that the aggregate carrier frequency for arRP alleles may be as high as 10% [10, 11]. Compared to X-linked and autosomal recessive variants, autosomal dominant disease has a less severe natural history, with a later onset [1].

Ophthalmoscopic Features

The classically described fundus appearance of RP includes intraretinal pigment migration causing a "bone spicule" appearance, mottling and granularity of the retinal pigment epithelium (RPE) that is often more marked in the mid-periphery, attenuated retinal vessels, and optic nerve head pallor ("waxy pallor") [1]. Fundus changes are often present in both eyes and exhibit a high degree of symmetry. Other ocular manifestations of RP include posterior subcapsular cataract, epiretinal membrane, and cystoid macula edema (CME) (Fig. 3.1).

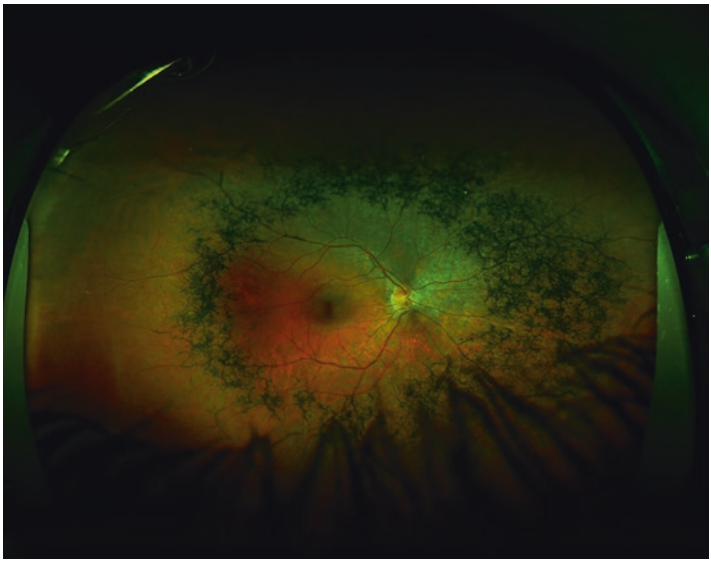

Fig. 3.1 Fundus photo of retinitis pigmentosa showing mid-peripheral "bone spicule" pigmentation

Imaging and Other Useful Tests

Perimetry and Electrophysiological Testing

Visual fields play an important role in the diagnosis of RP and also enable quantification of the change in visual deficit experienced by the patient. The earliest defects on visual fields for most RP patients, as assessed by kinetic perimetry, are relative scotomata in the mid-periphery between 30 and 50 degrees from fixation. These gradually enlarge and coalesce to form the classical peripheral ring scotoma.

The electroretinogram (ERG) measures the electrical potential generated by rods and cones after a light stimulus and is another important component in the diagnosis of RP. Karpe in 1945 first reported that the ERG was "extinguished" in RP [12]. In early stages of the disease, there is reduction in rod-specific b-wave amplitudes, but implicit time can be either prolonged or normal. Patients with advanced stages may have a non-detectable ERG. Berson et al. found that patients lost an average of 16–18.5% of remaining ERG amplitude to bright white flashes (a mixed rod-cone response) per year in the natural course of RP [13].

Fundus Autofluorescence

Fundus autofluorescence (FAF) uses a scanning laser ophthalmoscope to stimulate intrinsically autofluorescent molecules of lipofuscin to visualize the RPE [14]. In RP, FAF typically shows a perifoveal ring of increased autofluorescence within the macula, which demarcates the border between functional and dysfunctional retina. This border of increased autofluorescence appears to correlate with functional status as measured by pattern ERG, multifocal ERG, and microperimetry [15, 16]. The area outside the ring has also been correlated with anatomical changes, as seen on OCT, of disrupted inner segment-outer segment (IS/OS) junction and loss of outer nuclear layer (ONL) thickness (Fig. 3.2) [17, 18].

Fluorescein Angiography

Fluorescein angiography in patients with RP shows hyperfluorescence in areas of RPE atrophy and frequent abnormalities of the blood-retinal barrier at the level of the RPE. CME is a significant cause of early loss of visual acuity in RP, and these patients may exhibit a petaloid pattern of macular hyperfluorescence [19], but in RP, CME can also occur without evidence of dye leakage on fluorescein angiography (e.g., Goldmann-Favre syndrome, aka enhanced S-cone syndrome).

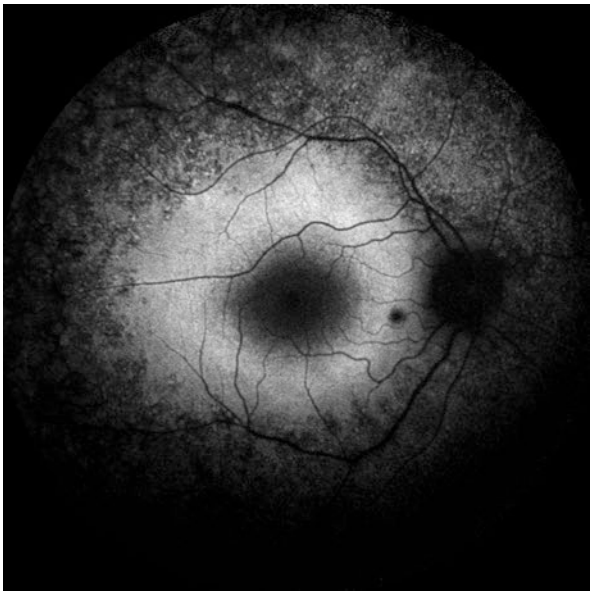

Fig. 3.2 Fundus autofluorescence image in retinitis pigmentosa showing the demarcation between a functional macula area and dysfunctional (hypo-autofluorescent) mid-peripheral retina

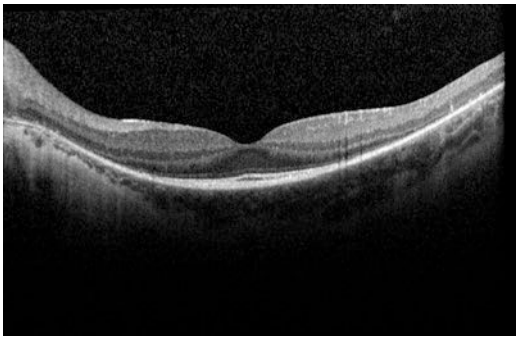

Fig. 3.3 Optical coherence tomography in retinitis pigmentosa showing perifoveal outer retinal degeneration

Optical Coherence Tomography (OCT)

OCT is a highly sensitive imaging modality that provides detailed anatomical assessment of the macula in patients with RP with resolution on the order of 3–7 μm. Common findings include decreased thickness of the outer nuclear layer (ONL), loss of the external limiting membrane and IS/OS junctions, and intraretinal cystic spaces. OCT findings have been correlated with visual defects as measured by visual fields, multifocal ERG, and microperimetry [20, 21]. There may also be retinal thickening due to CME and inner retinal distortion due to epiretinal membrane (Fig. 3.3) [22].

Differential Diagnosis

Both inherited and acquired retinal conditions may be confused with RP. Differentiation of RP from its phenocopies is important because these latter conditions bear important genetic and prognostic differences. Examples of important phenocopies to consider in patients with an inherited pigmented retinopathy include cone-rod dystrophy/late cone dystrophy, and Leber congenital amaurosis (LCA)/severe early childhood onset retinal dystrophy (SECORD).

Acquired conditions can produce extensive chorioretinal atrophy that may also resemble advanced RP. These include (1) retinal inflammatory diseases, such as rubella retinopathy, syphilis, toxoplasmosis, cytomegalovirus, and herpetic retinopathy; (2) paraneoplastic retinopathies, such as carcinoma-associated retinopathy (CAR) and melanoma-associated retinopathy (MAR); (3) drug toxicity involving chloroquine, hydroxychloroquine, thioridazine, chlorpromazine, or quinine; (4) traumatic retinopathy, and (5) miscellaneous conditions, such as diffuse unilateral subacute neuroretinitis (DUSN) and retinopathy associated with Zika virus infection [23]

Leber Congenital Amaurosis

Clinical Characteristics

Leber congenital amaurosis (LCA) encompasses a group of disorders characterized by severe progressive retinal dystrophy appearing at birth. Its prevalence ranges from 1:50000 to 1:100000. Although rare, LCA accounts for at least 5% of all inherited retinopathies [24].

In 1869, Theodor Leber defined LCA as a congenital form of retinitis pigmentosa with severe visual loss at or near birth, wandering nystagmus with the absence of visual fixation, poor pupillary reflexes, either normal or abnormal fundus appearance, and autosomal recessive inheritance [25]. The oculodigital sign, where infants press on the exterior of their eye with their fingers or knuckles to elicit retinal stimulation, is also a classic feature as described by Franceschetti and Dieterle [26]. Vision is typically poor, ranging from 20/200 to no light perception.

Systemic abnormalities are frequently encountered with LCA. Neurological abnormalities are the most common association, with up to 20% of patients developing mental retardation [27, 28]. Other systemic defects include deafness, renal impairment, infantile cardiomyopathy, hepatic dysfunction, and skeletal changes.

Genetic Association and Pathophysiology

LCA is not a single entity but a group of disorders, with mutations in at least 22 different genes already identified. The genes implicated in LCA are expressed preferentially in the retina or RPE. Their putative functions are diverse and include vitamin A metabolism (*RPE65*), phototransduction (*RetGC1/GUCY2D*), retinal

embryonic development (*CRX*), protein trafficking (*AIPL1* and *RPGRIP1*), photoreceptor cell structure (*CRB1*), and G protein trafficking (*CEP290*) [29]. LCA is generally inherited in an autosomal recessive fashion, although rarely it may be autosomal dominant (*CRX* or *IMPDH1* mutations) [30, 31].

Ophthalmoscopic Features

The fundus examination in LCA may be normal or show diffuse RPE granularity and retinal vessel attenuation. Other abnormalities that have been described in LCA include yellow retinal flecks, "salt and pepper" retinopathy, retinitis punctata albescens, and nummular pigment clumps [1, 32, 33]. Anterior segment abnormalities such as keratoconus [34] and cataract also may be present.

Imaging and Other Useful Tests

Fundus Autofluorescence, Fluorescein Angiography, and OCT

Fundus autofluorescence in LCA varies depending on the pathologic genotype. For instance, patients with *GUCY2D* mutations show a normal distribution of autofluorescence throughout the fundus, while those with *RPE65* mutations often show a severely reduced panretinal autofluorescence signal [29, 35].

Findings on fluorescein angiography are usually nonspecific, although retinal vascular attenuation is commonly found in cases of LCA with widespread retinal atrophy. Also, OCT may be normal in LCA or demonstrate macular atrophy depending on disease severity.

Psychophysical and Electrophysiological Testing

The ERG in patients with LCA is characteristically non-recordable or extinguished [36]. However, it is possible to have a small ERG signal early in the disease process, and the presence of a small signal should not preclude its diagnosis [24]. ERG testing in infants less than 1 year of age, and follow-up testing to achieve more robust recordings as the child grows older is advised to help differentiate LCA from other causes of congenital blindness, such as early onset RP, albinism, complete and incomplete achromatopsia, and complete and incomplete congenital stationary night blindness [37, 38].

Differential Diagnosis

Several entities may resemble LCA including early onset RP, congenital stationary night blindness (CSNB) [39], early infantile neuronal ceroid lipofuscinosis [40], Senior-Loken syndrome, and Saldino-Mainzer syndrome [1, 41].

Choroideremia

Clinical Characteristics

Choroideremia was first described by Mauthner in 1872 and causes a progressive, generalized retinal degeneration [42]. Its estimated prevalence is 1:50,000 and shows X-linked inheritance [43, 44]. The symptoms of choroideremia are similar to retinitis pigmentosa in that patients develop early loss of peripheral vision in their first and second decades of life, followed by loss of central vision later in life. Roberts et al. described a slow rate of visual acuity loss in 115 males with choroideremia with the retention of central visual acuity until the seventh decade [45]. A history of defective dark adaptation that manifests as poor visual function in dim illumination is commonly the first symptom. Carrier females in most instances do not experience significant visual symptoms.

Genetic Association and Pathophysiology

Choroideremia is an X-linked recessive rod-cone dystrophy. The genetic defect involves the *CHM* gene localized to the long arm of the X chromosome (Xq21) [46, 47]. This gene encodes geranyl-geranyl transferase Rab escort protein-1 (REP-1), which is localized to the RPE [44]. The REP-1 protein facilitates posttranslational modification of Rab proteins, which regulate intracellular trafficking in both RPE cells and photoreceptors and are thought to have a putative role in the removal of outer segment disc membranes by the RPE [48].

Ophthalmoscopic Features

Fundus findings in choroideremia typically undergo a characteristic progression from a fine, retinal pigment mottling in the mid-periphery with the underlying choroid appearing normal, to eventual loss of the choroid with bare exposure of sclera. No pigment migration is seen. The chorioretinal atrophy begins more peripherally and gradually encroaches on the macula. Most patients preserve their central visual acuity until late in the disease.

Carrier females of X-linked choroideremia show milder fundus changes than those observed in affected males. Typically there is mid-peripheral subretinal pigment mottling, classically described as a “moth eaten appearance” that may extend to the macula with progression. Intraretinal pigment migration is typically not found.

Imaging and Other Useful Tests

Fundus autofluorescence typically reveals loss of fluorescence in areas of chorioretinal atrophy and interspersed areas of persistent hyper-autofluorescence [49]. Carrier females may also demonstrate patchy areas of autofluorescence loss [50].

Fluorescein angiography reveals loss of the choriocapillaris throughout the fundus, even in patients with normal fundoscopy. Larger choroidal vessels appear unaffected, and the macula may also show a relatively preserved island of choriocapillaris. In female carriers, the fluorescein angiogram shows minimal, if any, disturbance of the choriocapillaris [51].

OCT accurately images the profound histopathological changes seen in choroideremia. In areas of affected retina, there is marked thinning of both outer retinal structures and the choriocapillaris. Outer retinal tubulation occurs commonly, signifying end-stage photoreceptor degeneration [52].

The ERG may be normal early in the course of the disease when only a few focal lesions are present [53]. However, the ERG becomes undetectable in end-stage disease. Electro-oculogram (EOG) readings, which reflect RPE function, show an abnormally low light-peak to dark-trough ratio. Dark adaptation testing shows an initial abnormality of the rod portion of the dark adaptation curve, with later involvement of the cone portion of the curve [1].

With the progressive development of equatorial and peripapillary choroidal vascular atrophy, formal perimetry shows corresponding diminished equatorial retinal sensitivity, the development of ring scotomata, and an enlarged blind spot. Gradual deterioration in vision occurs, and patients are ultimately left with less than ten degrees of visual field by their fifth and sixth decades.

Differential Diagnosis

The differential diagnosis of choroideremia includes gyrate atrophy, thioridazine (Mellaril) retinal toxicity, Bietti crystalline dystrophy, and RP.

Central Degenerations

Age-Related Macular Degeneration

Clinical Characteristics

Age-related macular degeneration (AMD) is a chronic progressive disease of the central macula and is the leading cause of irreversible visual loss among the elderly population in the developed world [54]. The prevalence of AMD increases exponentially with age [55]. The Australian Blue Mountain Eye Study showed the incidence of late AMD (geographic atrophy and neovascular AMD) to increase from 0% for those younger than 60 years, to 5.4% for those >80 years [54].

Patients with early AMD are usually asymptomatic, although patients may progress to exudative (neovascular) and non-exudative (atrophic) forms of the disease, both of which result in visual loss. Patients with neovascular AMD often report a sudden deterioration in their central vision, with metamorphopsia and the development of

a central scotoma. In atrophic AMD, loss of vision develops more gradually, over many years.

Genetic Association and Pathophysiology

The pathogenesis of AMD is incompletely understood. As a complex multifactorial disease, it is thought that a number of genetic, systemic, and environmental factors play a role. Aging itself appears to be the major risk factor. Biochemical, histological, and genetic studies have indicated several pathways in AMD pathogenesis, including malfunction of the complement system, chronic low-grade inflammation, oxidative damage, and excessive accumulation of lipofuscin [56].

Major progress has been made in elucidating the genetic basis of AMD through the identification of two common gene variants on chromosome 1 and 10, together accounting for almost 50% of cases. On chromosome 1, a strong association has been shown with risk variants in the complement factor H (CFH) gene [57–60], as well as lesser associations with the complement component 2/factor B [61], component 3 (C3), and complement factor I genes [62]. These findings highlight the importance of aberrant complement activity in AMD pathogenesis. The second major AMD risk locus includes two genes, the age-related maculopathy susceptibility 2 gene and the high temperature requirement factor A1 gene [63, 64]. The pathophysiologic significance of these mutations has not been established definitively.

Ophthalmoscopic Features

Drusen are considered the phenotypic hallmark of AMD, but are not pathognomonic of AMD [65]. Classically, drusen are focal deposits of extracellular material located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane. Funduscopically, they appear as yellow nodules, located primarily in the area centralis but also in the mid-periphery, and may occur as discrete lesions, in clusters, or as confluent drusenoid pigment epithelial detachments.

Drusen size has been correlated with visual prognosis in AMD. Small drusen ($\leq$63 μm) are considered part of normal aging, whereas the development of medium sized drusen (63–125 μm) without associated retinal pigment abnormalities represents "early AMD." The presence of any AMD-related pigmentary abnormalities and/or large drusen (>125 μm) has been classified as "intermediate AMD." The development of macular geographic atrophy (GA) signifies "advanced (non-exudative) AMD."

Besides the classic drusen phenotype described above, other drusen varieties have been described and have been shown to hold special pathophysiologic and prognostic importance. For instance, cuticular drusen, formerly known as basal laminar drusen, as described by Gass in 1977, appear as small (25–75 μm) round subretinal pigment epithelial nodules, often beginning as randomly scattered lesions

in the macula area [66]. They are found in approximately 10% of the AMD patients spectrum and have an association with mutations in the complement factor H gene (CFH Y402H) [67]. Structurally, they have a cuneiform appearance and have reflectance properties on OCT and fluorescein angiography that distinguish them from typical "soft" drusen [68].

Reticular (pseudo-) drusen are another important drusen species [69, 70], and their presence has been identified as an important risk factor for progression to end-stage AMD, including geographic atrophy and retinal angiomatous proliferation (RAP) [71, 72]. Reticular drusen appear as a network of multiple yellow irregularities, 50–250 μm in size. Histopathologic and OCT studies have localized the accumulation of material in reticular drusen to the area between the ellipsoid zone and the RPE apical surface [73, 74].

Retinal pigment epithelial changes in AMD result from RPE displacement, migration, and degeneration. Any hyper- or hypo-pigmentary abnormality associated with medium or large drusen is a defining feature of "intermediate AMD." Geographic atrophy meanwhile appears as a sharply demarcated area with depigmentation and enhanced visualization of deep choroidal vessels and may occur as unifocal or multifocal lesions.

Choroidal neovascularization (CNV) is the ingrowth of pathological new blood vessels from the choriocapillaris through Bruch's membrane into the sub-RPE and/or subretinal space. Leakage of plasma or blood into the surrounding tissue is characteristic, with eventual evolution to a fibrovascular scar [56].

Imaging and Other Useful Tests

Fluorescein Angiography

Using fluorescein angiography (FA), the morphology of choroidal new vessels (CNVs) was classified as "classic" or "occult." Classic CNVs show early hyperfluorescence and dye leakage and well-defined margins whereas occult CNVs later hyperfluorescence and may demonstrate either well-defined or poorly defined borders. Originally the differences in leakage pattern were assumed to be due to the anatomic location of the CNVM, with the classic form being associated with a subretinal (Type II) lesion and the occult form with a sub-RPE (type I) location. However, clinicopathologic studies have demonstrated a lack of strong correlation between the fluorescein angiogram pattern and anatomic localization of the CNVM [75].

For non-neovascular changes, classic small drusen exhibit focal hyperfluorescence in the late stage of the angiogram due to staining and larger drusen can vary in their appearance, depending on the biochemical composition of the druse [76]. FA is particularly useful in identifying cuticular drusen, as these fluoresce discretely during the early arterio-venous phase and produce a characteristic "stars-in-the-sky" appearance. In contrast to cuticular drusen, reticular drusen appear as a network of small discrete hypofluorescent lesions, especially in the late angiogram

frames. Finally, GA typically presents with well-defined hyperfluorescence due to staining of the exposed deep choroid and sclera (window defect).

For the diagnosis of neovascular AMD variants such as polypoidal choroidal vasculopathy (PCV) and RAP, indocyanine green angiography may provide anatomical information that is difficult to appreciate with FA.

Optical Coherence Tomography

OCT imaging allows precise visualization of fibrovascular structures associated with neovascular AMD and also enables serial assessment of intraretinal and subretinal fluid. Because clinicians rely heavily on the presence of intra- and subretinal fluid to make decisions about the need to treat conditions associated with retinal edema (e.g., CNV, macular edema associated with diabetes mellitus or retinal vein occlusion) and because the anatomical resolution of OCT for detecting edema is much greater than that of FA, OCT has become an indispensible tool in assessing therapeutic efficacy in the treatment of neovascular AMD.

Geographic atrophy on OCT is characterized by the loss of the inner part of the RPE/Bruch's membrane complex as well as outer retinal layers, including the outer nuclear layer, the external limiting membrane, ellipsoid zone, and inter-digitation zone. In the area surrounding GA, various associated micro-architectural alterations may be present including drusen and pigment migration [77].

Fundus Autofluorescence

Fundus autofluorescence (FAF) does not generally allow clear distinction of most AMD-related drusen because drusen exhibit a similar signal compared to the background [78]. One exception is large confluent drusen, which show a mildly increased signal. Both cuticular and reticular drusen are identified as areas of ill-defined autofluorescence centered by punctate hypo-autofluorescent lesions. In contrast to this, geographic atrophy appears as a well-demarcated lesion with decreased FAF signal, due to RPE atrophy and thus loss of intrinsic fluorophores (Fig. 3.4).

Normal, increased, or decreased focal RPE autofluorescence have all been observed in neovascular AMD [56]. Sometimes the CNVM identified with FAF may extend beyond the edge of the lesion defined by fluorescein angiography, which is postulated to be due to a reactive proliferation of RPE cells surrounding the CNVM [79].

Electrophysiological Testing

The full-field electroretinogram (ERG), which reflects panretinal cone and rod function, is generally not affected in AMD. Multifocal and pattern ERG, which are more selective measures of macula function, can become abnormal in early AMD

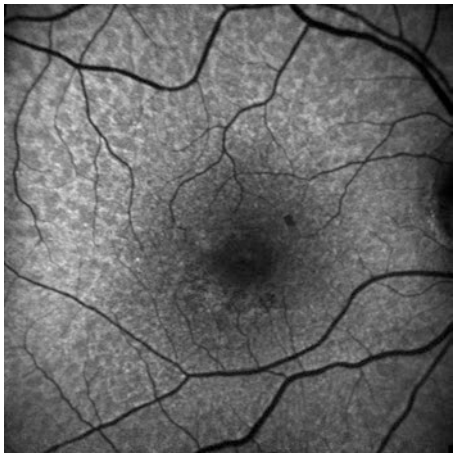

Fig. 3.4 Fundus autofluorescence image of reticular drusen

but are of limited differential diagnostic value. The electro-oculogram (EOG), which reflects global RPE function, is normal in AMD although it is useful in differentiating the late, atrophic forms of Best disease from AMD, as the EOG in Best disease is markedly abnormal.

Differential Diagnosis

The differential diagnosis for AMD varies depending on the presence or absence of CNVs and below has been divided into non-exudative and exudative forms:

- Non-exudative AMD: Pattern dystrophy, Stargardt disease, atrophic forms of Best disease, drug toxicity (e.g., hydroxychloroquine and chloroquine).
- Neovascular AMD: PCV [80], adult onset vitelliform macula dystrophy, central serous chorioretinopathy, idiopathic CNV, Sorsby fundus dystrophy, myopic choroidal neovascularization, CME, macular telangiectasia, and other causes of CNV (e.g., angioid streaks).

ABCA4 (Stargardt) Dystrophy

Clinical Characteristics

Stargardt disease is a hereditary retinal dystrophy that is usually diagnosed within the first two decades of life, and it is the most common form of inherited juvenile macular degeneration. It classically presents in childhood with decreased central vision. Both Stargardt disease and fundus flavimaculatus share a common genetic

defect in the *ABCA4* gene, although fundus flavimaculatus generally represents a milder phenotype with later disease onset and better prognosis. Stargardt disease has an estimated prevalence of 1:8000–1:10000 [81], although the true prevalence could be higher because the carrier frequency for ABCA4 mutations is thought to be as high as 1 in 20 [10].

Genetic Association and Pathophysiology

Homozygous or compound heterozygous mutations in the gene encoding the photoreceptor-specific, ATP-binding cassette transporter A4 (ABCA4) are responsible for Stargardt disease [82]. Over 600 disease-causing mutations in the ABCA4 gene have been identified, of which the three most common mutations account for less than 10% of the disease phenotypes [10].

ABCA4 defects lead to the intracellular accumulation of N-retinylidene-N-retinylethanolamine (A2E) in RPE cells [83, 84]. A2E, a component of lipofuscin, is cytotoxic to the RPE in high concentrations [85]. Studies in ABCA4 knockout mice modeling Stargardt disease have shown that oxidative stress, complement activation, and downregulation of protective complement regulatory proteins potentially underlie the pathophysiology of this retinal dystrophy [84].

ABCA4 retinopathy may present with a wide spectrum of phenotypic variability manifesting as AMD in heterozygous (monoallelic) carriers, autosomal recessive cone-rod dystrophy, and autosomal recessive retinitis pigmentosa (arRP) [86].

Ophthalmoscopic Features

The fundus exam in Stargardt disease shows irregular pisciform flecks scattered throughout the posterior pole, which may extend to the mid-peripheral retina. Extensive chorioretinal atrophy may also be seen. Development of CNVs, though, is rare.

Stargardt disease has been described as proceeding through four disease stages [87]. Stage 1 is confined to the fovea or para-foveal macula, with RPE pigment changes and outer segment thinning. A discontinuous ring of flecks approximately 1 disc diameter in size often encircles the fovea, and both the ERG and EOG are normal. The retinal flecks become more widespread in Stage II, and subtle changes in the ERG and dark adaptation begin to emerge. Stage III demonstrates resorption of retinal flecks and atrophy of the posterior pole choriocapillaris, with progression to extensive choriocapillaris and RPE atrophy in Stage IV.

Imaging and Other Useful Tests

Fundus Autofluorescence

FAF provides a qualitative assessment of the buildup and distribution of lipofuscin in ABCA4 disease and allows detection of changes in the function of the RPE before these can be appreciated on fundoscopy. Retinal flecks in Stargardt disease correspond to areas of focal hyper-autofluorescence while RPE atrophy produces hypo-autofluorescence due to the absence of fluorophores. A relatively preserved autofluorescent signal of the fovea is seen in up to 50% of Stargardt cases, indicating subfoveal RPE sparing (Fig. 3.5) [56].

Fluorescein Angiography

At least 80% of Stargardt patients have a "silent" or dark choroid on fluorescein angiogram [88] due to A2E accumulation in the RPE, which in turn masks the background choroidal fluorescence.

Optical Coherence Tomography

OCT imaging shows hyper-reflective thickening of the RPE layer in Stargardt disease, which distinguishes it from sub-RPE drusen in AMD. Loss of the inner segment-outer segment (IS-OS) junction correlates with atrophy seen on fluorescein

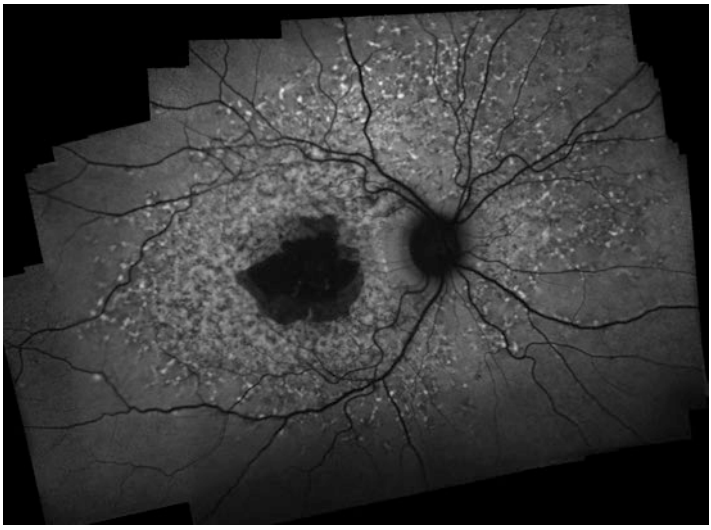

Fig. 3.5 Fundus autofluorescence image in ABCA4 retinal dystrophy showing macula hyper-autofluorescent pisciform retinal flecks and a central area of hypo-autofluorescence corresponding to photoreceptor and RPE degeneration

angiography and FAF [89], and widespread thinning of the inner and outer retina and RPE are seen with more advanced retinal disease.

Electrophysiological Testing

Three subgroups of Stargardt patients have been defined on the basis of electrophysiology—Group 1 patients have a normal full-field ERG; Group 2 patients have impaired photopic function but preserved scotopic responses; and Group 3 patients have both an abnormal scotopic and photopic ERG and have the worst visual prognosis [90].

Differential Diagnosis

Stargardt disease, especially late-onset forms of the disease, may be confused with AMD due to the presence of yellowish flecks, chorioretinal atrophy, and, rarely, CNVs. However, the retinal flecks seen in Stargardt disease are more irregularly shaped than drusen and show intense hyper-autofluorescence with a surrounding halo of decreased FAF signal. Stargardt patients also show the characteristic "dark choroid" on fluorescein angiography (particularly in a peripapillary distribution), which is not typically seen in AMD.

Best Disease

Clinical Characteristics

Best disease is an early-onset form of vitelliform macular dystrophy and was first described by Franz Best in 1905. The age at onset of central visual loss is highly variable and may range from the first to the sixth decade, but most patients are symptomatic before the age of forty [56]. It has been characterized as progressing through five stages, although some controversy exists regarding the chronological order of these stages [91]:.

- Previtelliform.
- Vitelliform.
- Pseudohypopyon.
- Vitelliruptive.
- Atrophic.

Vision is usually good during the initial stages of the disease. However, vision typically decreases during the vitelliruptive or "scrambled egg" stage, and chorioretinal atrophy develops over time. The development of CNVs occurs in 2–9% of

patients with Best disease, and these CNVs tend to show a relatively benign and self-limiting course [91–93].

Genetic Association and Pathophysiology

Best disease is an autosomal dominant maculopathy with incomplete penetrance and variable expression [10]. It is caused by a defect in the *BEST1* gene, previously known as *VMD2*, which encodes bestrophin-1, a calcium-dependent chloride channel found in the RPE [94]. Mutations in the bestropin-1 protein are thought to cause defective light-induced chloride efflux, leading to the accumulation of lipofuscin and fluid within and beneath the RPE. This causes the characteristic bilateral "egg-yolk" appearance of the macular. Prolonged neuroretinal detachment and lipofuscin overload of the RPE eventually leads to photoreceptor and RPE dysfunction.

Ophthalmoscopic Features

The characteristic yellow "egg-yolk" appearance of macular deposits seen in Best disease may sometimes be confused with adult foveomacular vitelliform dystrophy, which usually develops much later in life, between 30 and 60 years of age and, in some cases, is due to a mutation in the peripherin-2/rds gene [95]. The atrophic stage of Best disease may be followed by development of CNVs, which often is associated with the worst visual prognosis.

Imaging and Other Useful Tests

Optical Coherence Tomography

OCT has shown that the vitelliform material in Best disease accumulates between the RPE and retina, suggesting that the material is composed of shed photoreceptor outer segments [96]. In more advanced lesions, hyper-reflective subretinal scars and intraretinal edema also may be seen.

Fundus autofluorescence

The lesions in Best disease show areas of intense hyper-autofluorescence that correspond to the vitelliform material [96]. Areas of scarring and atrophic RPE show decreased autofluorescence (Fig. 3.6).

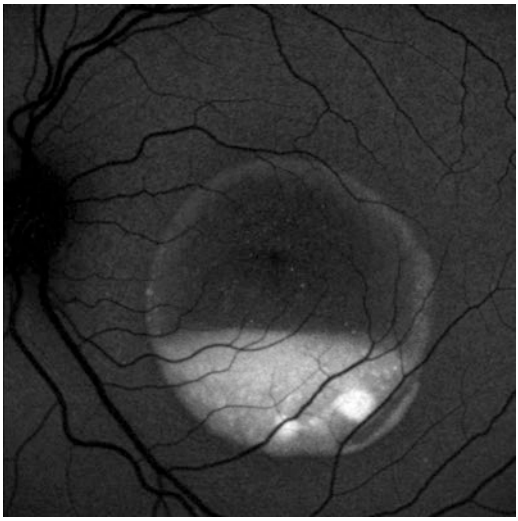

Fig. 3.6 Fundus autofluorescent image of pseudohypopyon stage of Best disease

Fluorescein Angiography

The early phases on FA show blocked background fluorescence by lesions with large amounts of vitelliform material. In other cases, the lesions may show mild hyperfluoresence due to RPE window defects and/or staining [96].

Electrophysiological Testing

The pathognomonic feature of Best disease and other *BEST1*-gene-related diseases is the absence of a normal light-rise on the EOG, reflected by a markedly diminished Arden ration (<1.5) in both affected patients and carriers.

Differential Diagnosis

Best disease may be differentiated from AMD due to its younger age of onset, the presence of central vitelliform lesions without surrounding drusen, marked autofluorescence changes within the Best lesions, and a markedly abnormal EOG. The autosomal dominant inheritance pattern also is much more typical of Best disease than AMD.

Conclusion

Harnessing the recent cost reduction in whole genome sequencing, human disorders are stratified to the gene level and advancing "pharmacogenomics," or the science of prescribing "the right drug for the right patient at the right dose." For example, *ABCA4* gene sequencing is a prerequisite for the ongoing Stargardt pharmacotherapy trials. Precision medicine determines each individual's "genetic profile"—his or her entire DNA code—and uses that data to identify the best course of treatment. Precision medicine suggests that the cure to a disease is not a generic, "one-size-fits-all" model, but rather differs from person to person. The cure exists at the most basic, molecular level of the human body: the genes.

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Chapter 4
Degenerative Retinal Diseases: Cell Sources for Cell-Based Therapy

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Retinal Degenerative Diseases

Thanks to recent advances in molecular cell biology, research in the field of retinal degenerative disease has expanded considerably. These advances have allowed us to elucidate underlying pathophysiological mechanisms, thereby helping us to propose rational therapeutic approaches such as gene or cell therapy. Retinal degenerative diseases tend to be progressive and irreversible. They can be hereditary (e.g., retinitis pigmentosa [RP] or Stargardt disease [STGD]) or acquired (e.g., hydroxychloroquine toxicity). Most have no effective treatment and will ultimately lead to blindness. Age-related macular degeneration (AMD), RP, and STGD are the most frequent, being responsible for blindness in a significant number of people [1]. Estimates from the World Health Organization indicate that there are approximately 161 million visually impaired people worldwide, of whom 37 million are clinically blind, with an annual incidence of 1 to 2 million cases [2, 3].

AMD is a complex disorder with a multifactorial etiology that affects the macular region of the retina and involves the retinal pigment epithelium (RPE), the Bruch membrane (BM), and the choriocapillaris. In a subset of patients, it is associated

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with progressive and irreversible loss of central vision [4]. AMD accounts for 8.7% of all cases of blindness and is the most common cause of blindness in people older than 60 years. This incidence is expected to increase due to the overall improvements in life expectancy [2]. AMD is most prevalent in Europeans (11.2–13.2%), in whom the geographic atrophy (GA) subtype accounts for around 1.1% [2].

With age, the retina accumulates deposits of abnormal extracellular material known as drusen. Under pathophysiological conditions of cellular inflammation, oxidative stress, and angiogenesis, these deposits may compromise the structure and function of the RPE–BM–choriocapillaris complex as well as reflecting the compromised function of these tissues. In the exudative form of AMD (known as wet or neovascular AMD [nAMD]), new leaky blood vessels from the choroid grow into the sub-RPE and subretinal spaces, probably as a homeostatic response to local tissue ischemia, causing extensive hemorrhage and edema that progressively disrupts visual function [5]. In the last decade, the visual prognosis of nAMD has improved due to the introduction of intravitreal anti-angiogenic drug treatments [6]. Nevertheless, the natural history still causes major structural alterations in the affected macular region, with RPE, photoreceptor, and neural connecting cells dying as a consequence [7]. GA (dry, atrophic, or non-exudative AMD) occurs when macular RPE cells and the overlying neurosensory retina and subjacent choriocapillaris degenerate with progressive impairment of central vision [4, 8]. Thus, the death of RPE cells and photoreceptors (rods and cones) has been documented in both clinical presentations of AMD, although rod loss seems to precede cone loss [4, 9]. Transsynaptic retinal degeneration also occurs in second-order retinal neurons, typically associated with dendritic sprouting.

RP and STGD usually cause symptoms and visual loss in childhood and young adulthood. RP affects 1.5 million people worldwide [1]. Recent advances in molecular genetics have enabled genes and biochemical pathways associated with disease pathogenesis to be identified and which underlie the monogenic and/or polygenic inheritance of these disorders [10, 11]. The RPE is essential for the function and survival of cones and rods, and its dysfunction or death has been observed in RP and STGD, for which multiple alterations in biochemical pathways and cellular mechanisms have been identified [10–12]. In photoreceptor cells, dysfunctional events in phototransduction, vesicle trafficking, lipid metabolism, transcription/RNA splicing, and synaptic function have been described. In RPE cells, by contrast, affected mechanisms include the visual cycle, phagocytosis, and membrane trafficking. Although many clinical trials are ongoing for these diseases, therapeutic options remain limited. Stem cell therapy, retinal prostheses, and optogenetics are emerging as therapeutic alternatives.

The use of stem cells for organ regeneration and tissue repair is currently a subject of great scientific interest. Ethical and practical obstacles with the use of human embryonic stem cells (hESC) have led to the use of somatic stem cells from adult human tissue, and these have been shown to provide an excellent alternative cell source [13, 14]. These cells have significant clinical advantages when used in cell therapy, including their ready availability, the ease of maintenance techniques for

in vitro cell culture, excellent proliferation rates for ex vivo expansion, and excellent in vitro plasticity [15]. However, despite the use of various cell types for cell-based retinal therapy in preclinical models, several challenges remain. Points that still need to be resolved include the correct differentiation of cells, the safety of the approaches used, the optimal techniques and systems of cell delivery, the cell production and normative issues, and how best to integrate cells with the receptor tissue so that appropriate synapse formation occurs. Once implanted, the cells can preserve and/or restore vision by altering the environment ("rescue therapy") or by replacing damaged tissue ("replacement therapy"). Finally, cell survival in allogenic approaches depends not only on appropriate environmental cues but also on the immune tolerance of the host. Several cell-based therapies are being developed in clinical trials.

In this chapter, we provide a brief update on the different cell sources with potential for clinical use in the treatment of degenerative retinal disease in humans.

Sources of Stem Cells

During the last decade, improvements in the clinical use of progenitor cells have allowed a significant advance in the development of approaches aimed at the treatment of many conditions, including degenerative diseases of the retina. Various cellular sources can be used for diverse clinical applications, as well as for disease modeling and the screening of pharmacological agents.

By definition, stem cells are characterized by three general properties: the capacity for self-renewal (asymmetric cell division), their undifferentiated state, and the ability to differentiate toward various types of cells and tissues. Broadly, the progenitor potential can then be sub-classified into pluripotent stem cells, which can differentiate into any cell types within an organism (i.e., endoderm, ectoderm, mesoderm), or multipotent stem cells, which can only differentiate into a limited number of cell types that will give rise to certain tissues. Cells can also be classified by their origin: embryonic stem cells (ESCs) are obtained from approximately 30 cells of the inner cell mass of a blastocyst; fetal progenitor cells are derived from a variety of developing fetal tissues; and adult progenitor cells are derived from functional tissues of an adult organism. Finally, progenitor cells can be classified by the tissue from which they are derived, e.g., neuronal origin progenitor cells (from neuroectodermal tissue) and nonneuronal origin progenitor cells (from non-neuroectodermal tissues) [13, 16].

Next, the main sources of progenitor cells used for photoreceptors and/or RPE recovery/replacement approaches will be briefly discussed as well as their current therapeutic applications.

Embryonic Stem Cells (ESCs)

The preclinical evidence and results observed in clinical trials demonstrate that the use of human embryonic stem cells (hESCs) is safe for clinical use in replacement therapies for degenerative retinal disease in humans.

The first in vitro cultures of hESCs were obtained from the inner cell mass of developing blastocysts (~5-day-old preimplantation embryos) and maintained in a coculture approach (feeder layer) with murine embryonic fibroblasts and non-defined complete culture media containing serum and extracts, rich in growth factors, polypeptides, and proteins that stimulate cell growth. These methods aimed to maintain the viability of cells [17]. Under these conditions, hESCs initially preserved their undifferentiated characteristics, with an extensive capacity to develop into various cell types (pluripotency), and maintained their capacity for self-renewal in vitro [18]. However, there have always been concerns regarding tumor formation and immunological rejection because these cells are highly undifferentiated, allogeneic, and pluripotent. Improved understanding of the behavior and in vitro metabolic needs of hESCs has led to the adaptation of different approaches for their maintenance in culture, with defined cellular media and xenobiotic-free culture protocols that enhance their clinical applicability [19, 20].

Since the first investigations with hESCs, many studies have been undertaken to develop protocols for the induction of these cells to neural retinal lineages or RPE cells [21]. Bone morphogenic protein (BMP), insulin-like growth factor 1 (IGF-1), and the Wnt-β catenin signaling pathway are adjuvants for effective derivation into neural retinal progenitor cells [22, 23]. Functional RPE cells were first derived from hESCs by spontaneous differentiation [24] and later by faster and more effective protocols [25, 26], but more recently an efficient method that uses xenobiotic-free culture media and cellular growth on human laminin-521 substrate [27]. In a clinical-grade defined culture medium, without fibroblast growth factor (FGF) and transforming growth factor β (TGF-β), and subjected to a short initial treatment with RhoA/Rho-kinase (ROCK), hESC cultures were shown to induce a robust derivation toward RPE cells, obtaining fully differentiated and functional cells with a 9-week derivation protocol.

Several studies in experimental models of retinal degeneration have evaluated the viability and tolerability of RPE cells derived from hESCs by subretinal transplantation, by implanting the cells either in suspension or in sheets of a monolayer of polarized cells. In vivo imaging indicated survival of some pigmented cells on host BM with migration of some cells into an epiretinal location, and clinical assessment suggested improvement in some visual parameters [1, 28, 29]. Unfortunately, retinal neurons derived from the hESCs do not exhibit the same integration efficiency in host tissue after subretinal transplantation [14]. An as yet unresolved question about ESCs is the possibility that their secretome has paracrine properties. Unlike mesenchymal stem cells (MSCs), which have a proven beneficial effect for the secretion of many trophic factors, this action has not been demonstrated for ESCs when used for degenerative retinal diseases [30]. However, ESC-derived human RPE do produce numerous growth factors and presumably can have paracrine effects [31].

Interestingly, under appropriate three-dimensional (3D) cell culture conditions, hESCs are able to differentiate and self-organize into 3D structures that histologically resemble a primitive optic vesicle [32, 33]. In addition, at more advanced stages of growth, they can generate stratified elements that mimic the neural retina. More recently, 3D stratified retinal organoids have been reproduced from hESCs, which have not required evagination of the optic vesicle to generate structures such as the neural retina. This derivation protocol has allowed the review of ocular morphogenesis and maximization of the genesis of retinal elements such as photoreceptors (PRs) and RPE [34].

Based on preclinical results, phase I/II/III clinical trials have either been conducted or are underway with the aim of investigating the subretinal application of RPE cells derived from hESCs [35]. These studies have shown that these stem cell-derived RPE cells can survive in the host tissue and maintain some biological activity without signs of tumorigenesis [35, 36]. In some patients, there was limited evidence for hyperproliferation (e.g., pigmented epiretinal membrane formation). It is more difficult to judge whether there was immune rejection or not. There seemed to be no evidence of a robust inflammatory response, but immune rejection can also occur with minimal cellular infiltration and, in that case, would be difficult to detect clinically except with long-term follow-up of graft survival. One route of administration for ESC-derived RPE is subretinal delivery of cell suspensions (between $5x10^4$ and $2x10^5$ cells) [37]. At present, several clinical trials are testing the safety, tolerability, and functionality of RPE cells derived from the MA09 (hESCs) line with regard to their applicability to different human degenerative retinal diseases. The MA09 line was derived from single blastomeres from human embryos and expresses the pluripotency gene (Nanog, POU5F1-Oct4) and cellular markers (SSEA-3, SSEA-4; TRA-1-60; TRA-1-81) with a normal karyotype of 46(XX). Their ability to differentiate into endo-, meso-, and ectodermal tissues has been documented. Cells are cultured in hESC growth media on mouse embryonic fibroblast feeder-layer cells (source: Human Pluripotent Stem Cell Registry, https://hpscreg.eu/). Currently, this is the human embryonic cell line most used for the derivation of RPE cells for clinical use.

Fetal Stem Cells

Early investigations have demonstrated the technical feasibility of subretinal transplantation in different experimental models, as well as in humans with retinal degeneration. The PR and RPE cells, either as suspended cellular microaggregates in the form of dissociated tissue or as complete epithelial sheets of neurosensory retinal tissue with or without associated RPE, have been used with some success. There is some evidence that transplanting differentiated sheets of RPE may have survival and rescue advantages over RPE suspensions [38, 39]. In addition, these approaches provide evidence that the use of allogenic PRs generally does not elicit an immune response when implanted in the subretinal space, but that there is some risk of graft rejection for RPE [35].

Thanks to a better understanding of the maturation processes of the human retina during development, it is known that the process of differentiation and specialization of the PRs in the neurosensory retina occurs between the third and fifth months of gestation, making this the ideal period for obtaining cells from fetal tissue [40]. The use of retina-derived progenitor cells from fetuses (fetal retinal progenitor cells) has been developed from in vivo models in which post-mitotic PR precursor cells were observed to induce an acceptable degree of integration and some efficiency in restoring the functionality of the damaged retina [41–43]. However, additional data seem to indicate that relatively little integration of transplanted PR receptor precursors with host retina occurred [44–46]. Rather, cell fusion seems to have been the basis for improved PR survival in the host retina in these experiments.

The difficulties and ethical issues associated obtaining fetal tissue for clinical use should also be mentioned. In addition, despite the advances in the approaches used for cells and tissues of fetal origin, some caution must remain regarding the evaluation of the functional recovery of the damaged retina. An important point requiring clarification is whether true integration of the transplanted elements occurs in the recipient retina or whether there is only a neurotrophic effect on the tissue [42, 47]. In a phase I/II clinical trial that is currently in progress, the safety and efficacy of human fetal RPE cells transplanted in the subretinal space is being evaluated in patients with AMD.

Adult Stem Cells

Multipotent progenitor cells from adult tissue now represent an excellent alternative for cell therapies, where not only is replacement sought but also when tissue is needed for recovery in the incipient phases of the degenerative process. It has long been considered that the human retina, being a highly specialized and differentiated tissue similar to the central nervous system (CNS), lacks regenerative properties. However, some capacity for in vivo neurogenesis has been observed, with the identification of cellular subpopulations in the non-laminated margin of the peripheral retina and in the *pars plana* region of the ciliary body that express progenitor and neuronal molecular markers [48–51]. In addition, Muller cells may have the capacity to differentiate into PRs [52]. In this connection, Muller cells or the peripheral retinal margin in humans could represent a region in which cellular morphological maturation occurs in a way that recapitulates the normal development of retinal cells, opening up future perspectives for endogenous stimulatory therapies and optogenetic approaches [48, 51].

Neuronal Origin

Among the multipotent progenitor cells in adult tissue of neuronal origin, providing preclinical evidence that validates clinical use in humans, RPE stem cells, ciliary epithelium-derived progenitor (CEP) cells, and Müller progenitor cells are all candidates. Despite advances in our understanding of the characteristics and behavior

of such cells in vitro and in experimental models, their possible clinical application within the context of cell-based therapies for retinal degenerative disease has yet to be clarified. Uncertainties exist about their origin, the molecular mechanisms associated with differentiation events, and the possibility of large-scale ex vivo expansion for therapeutic application. Each of these issues must be studied to validate a cell's potential as a source of stem cells for clinical purposes [28, 53].

RPE Stem Cells

A unique characteristic of the RPE is its transdifferentiation capacity. This is a process by which the RPE loses its high degree of specialization and regains its so-called "stemness," allowing proliferation and differentiation into the various neuronal cell types to restore the neurosensory retina and RPE both anatomically and functionally. This exceptional spontaneous regenerative phenomenon has only been observed in some adult amphibians and, to a more limited extent, in some species of birds, occurring exclusively during embryonic development in the latter case [28, 53–55]. However, a very small subset of RPE cells with proliferative capacity has been demonstrated in the peripheral region of the RPE monolayer of some mammalian eyes [13, 53]. In adult human eyes, this cellular subpopulation has been isolated and cultured in vitro, and under certain conditions, differentiated into cells with characteristics of neural progenitors of the retina, thereby conferring some multipotent capacity [28, 56, 57]. These cells have also shown some capacity for dedifferentiation and redifferentiation into RPE, losing and recovering the expression of specific cellular and molecular markers [56, 57]. Their potential for differentiation into glial cells, ganglion cells, amacrine cells, and PRs also has been demonstrated [53].

An interesting fact is that the subpopulation of RPE stem cells, similar to other types of retinal progenitor cells, tend to present a very high clonal behavior with more undifferentiated cell phenotype when cultured in a neurosphere setting. These cells can differentiate into neuronal progeny of the retina and RPE, expressing early tissue markers of ocular tissue (Pax6 and Mitf), and, surprisingly, can also differentiate into mesenchymal lineages capable of inducing osteo-, chondro-, and adipogenesis [57]. This finding is consistent with the fact that RPE cells are neuroectodermal in origin [13, 53].

Coupled with the fact that these cells can be obtained and cultivated with some ease, we may consider that these are a theoretical source of allogeneic adult cells for cell replacement approaches in retinal degenerative diseases where there is a loss or deterioration of the RPE.

Ciliary Epithelium-Derived Progenitor Cells

As discussed above, CEP cells are a subpopulation with progenitor characteristics that are present at the periphery of the human retina and could induce events related to neurogenesis [48]. When isolated from the human retina, they at least have a high

clonogenic capacity from neurospheres in vitro [49], with relative ability to differentiate and express not only typical markers of progenitor cells (Chx10 and nestin) but also markers typical of adult retinal cells, such as those for RPE, PRs, and specialized neurons and interneurons (e.g., ganglion and horizontal cells) [28, 49, 58]. However, the molecular signaling events that trigger a differentiation stimulus in vivo are still unknown.

It seems that CEP cells are a subpopulation of neuroectodermic origin with some capacity for self-renewal, but with limited properties for authentic multipotent differentiation [58–60]. In this sense, one of the main points of controversy about the limitation of CEP cells for potential use as retinal stem cells lies in the question of their true cellular identity and the discrepancies related to their differentiation potential toward the various retinal cell types, mainly PRs, and their capacity to integrate in the receptor retina of experimental models [28, 53, 59, 60]. Although additional research may elucidate these issues and although the evidence suggests that human CEP cells may be considered retinal stem cells, there are other important problems regarding their clinical use, such as the low yield of differentiated terminal cells and their very low prevalence within the population of retinal progenitor cells. Moreover, although CEP cells can express various retinal progenitor markers in vitro, they appear to have limited potential for differentiation toward neural cell of the retina [28, 53].

Müller Progenitor Cells (MCs)

MCs are not typical stem cells because of their differentiated state and specific functionality. Nonetheless, they have elicited enthusiasm for the treatment of certain degenerative eye diseases because a subpopulation of MCs can acquire stem cell characteristics under specific conditions. In amphibians and birds, MCs can completely regenerate the neurosensory retina or produce neurons after a retinal insult [61]. This capacity for endogenous regeneration is more limited in mammals [62, 63], but the human retina does contain a small population of cells (about 1000 cells) that maintain their stem cell characteristics [49]. MCs are the main glial cell population of the retina, providing structural and metabolic support for neural and vascular cells [62, 63]. Moreover, MCs with progenitor cell properties have been isolated and propagated from adult human retinas [64]. Murine models and in vitro approaches have identified a human subpopulation of MCs with stem cell characteristics that can create retinal neurons, including PRs [50, 65].

Reparative mechanisms of retinal tissue from the glial elements imply complex molecular signaling mechanisms for which growth factors play a crucial role [63]. Thus, MCs do not have the ability to enter into the mitotic cycle spontaneously after retinal tissue damage, but can be stimulated to do so by specific growth factors [63]. Theoretically, if the retinal injury is localized, MCs located in the inner nuclear layer of the retina could be induced as a cell source for the replacement of damaged retinal tissue [55, 62, 63].

When seeded at very low density on a suitable extracellular matrix and with specific culture medium supplemented with retinoic acid and FGF, the MIO-M1 line of human Müller cells (CRALBP+/nestin+) possesses the ability to form neurospheres in vitro [66]. Under these conditions, the cells present a clonal capacity similar to that of progenitor cells, besides adopting an immortalized and stable cell line behavior through innumerable culture passes. MIO-M1 cells share several characteristics with neural stem cells, including the expressions of Sox2, Pax6, and Notch1 [64]. In experimental models, they can migrate and assume phenotypes similar to ganglion and PR cells once implanted in the subretinal space, indicating a certain regenerative capacity [53].

Thus, MCs can be stimulated after retinal tissue injury, can be isolated and proliferated in vitro, can migrate and differentiate into cells with neuronal retinal phenotypes in experimental models, and can integrate into retinal tissue.

Nonneuronal Origin

In the context of cell-based therapies for the replacement and/or recovery of degenerative pathology of the retina and optic nerve, the most significant advances in basic and translational research have been in embryonic, fetal, and neuronal cell sources. Although many studies have focused on neuroectodermal cell lines, other nonneural lineages have been the targets of numerous studies and some clinical trials that are currently underway. Cells of non-neuroectodermal origin, especially progenitor cells of adult tissue, are the most investigated.

The multipotent potential of these progenitor cells has not yet been fully elucidated, but knowledge regarding their behavior has certainly increased in the last decades. Because they are adult cells with proven stem-like characteristics, they are an attractive alternative stem cell source for the treatment of diseases of the retina and optic nerve [35, 67–69]. Among the main cellular sources, currently the most explored are mesenchymal stem cells (MSCs) of adipose tissue (AT-MSCs) and bone marrow (BM-MSCs), as well as umbilical cord-derived stem cells (UCSCs) [13]. These progenitor cells are derived from adult tissue originating from hematopoietic compartments (CD34+), represented by cell types from bone marrow (CD34+ and CD34−), the placental (CD133−), and white adipose tissue (CD34−). Therefore, there are no ethical issues with their production or application as autologous cells, with the latter already established in clinical practice. Their excellent capacity to differentiate into various cell types—though limited to neuronal tissue—means that the therapeutic effect of UCSCs arises from their neurotrophic properties via paracrine secretion [13, 35]. In this sense, they could be excellent alternatives for rescue therapy when there is early neuronal tissue degeneration, providing the neurotrophic and/or neuroprotective factors essential for the maintenance and functional preservation of tissue.

The immunomodulatory properties of MSCs make them an especially attractive source for clinical use. The results of several preclinical studies have the potential to be translated into clinical practice, but for rescue purposes, not replacement. At present,

for example, effective differentiation into RPE and PR cells has still not been demonstrated. However, MSCs can be obtained easily, expanded on a large scale, and preserved for long periods of time, allowing autologous use. These favorable clinical characteristics indicate an excellent safety profile, and their allogeneic use is now being explored (MSCs lack HLA-DR expression) [14, 70].

Adipose Tissue Mesenchymal Stem Cells (AT-MSCs)

AT-MSCs, also called adipose-derived stromal cells (ASCs), have several advantages over other MSC lines in adult tissue as an alternative source of stem cells. In particular, they are easily accessible, and the cellular performance is significantly greater in culture. AT-MSCs have been at the forefront of cell therapy and tissue engineering due to both their multipotent potential and their ability to be differentiated easily in vitro in multiple lineages, including adipocytes, osteoblasts, chondrocytes, and even cells of non-mesodermal origin. Similar to all MSC lines in adult tissue, but unlike ESCs, their use does not pose ethical problems. They can be isolated from small biopsies of adipose tissue or from the surgical residues of planned liposuction, offering potential for autologous or allogeneic application.

A critical point regarding AT-MSCs concerns the heterogeneity of the cellular subpopulations that can be isolated in white adipose tissue. This richness in cell populations, many of them with progenitor characteristics, may occur for several reasons, including differences in the isolation protocols, in the cell culture conditions, in the techniques used for liposuction, and in other idiosyncratic factors of the donor (e.g., body mass index, gender, and age). The heterogeneity of AT-MSCs decreases as their permanence increases under cultivation conditions, so it is recommended that they be used after obtaining passaging the cells in vitro [71]. The in vitro characterization of AT-MSCs was also a crucial point that could be solved by establishing a consensus among experts in the field [72]. The minimum criteria for cells to be considered MSCs are that they must: (1) present fibroblastic morphology with spontaneous adhesion to a plastic substrate (culture plate); (2) express ($\geq$95%) CD73, CD90, and CD105; (3) not express or minimally express ($\leq$2%) CD34, CD45, CD14, CD11b, CD19, CD79α, or HLA-DR; and (4) have in vitro differentiation capacity for osteogenesis, adipogenesis, and chondrogenesis lineages, which must be evidenced by specific culture stains [73].

AT-MSCs have very low immunogenicity and even show immunosuppressive properties, which suggested their potential value for immunomodulatory treatment in autoimmune diseases. In addition, their ability to produce and secrete trophic factors essential for various tissues has been demonstrated, making AT-MSCs highly relevant for clinical applications with broad therapeutic potential. Due to the metabolic versatility and high plasticity of AT-MSCs, it is important to assess their capacity for tumorigenesis and metastasis, either from the cellular proliferation of residual tumors or from the implanted cells themselves causing de novo carcinogenesis [74].

The ability of AT-MSCs to maintain in vitro differentiation toward cells with neuronal characteristics remains questionable, so this presently limits their clinical application for retinal cell replacement therapy. AT-MSCs demonstrated limited survival potential and integration when implanted in the host retina of experimental models. By contrast, the paracrine properties of AT-MSCs are well documented, suggesting the potential for rescue therapy in neurodegenerative conditions [14, 70, 75]. AT-MSCs express nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurothrophin-3 (NT-3), glial cell-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) at high concentrations, with BDNF and VEGF being expressed at higher concentrations than expressed by BM-MSCs. These trophic properties, which are neuroprotective and/or neuroregenerative, were tested in different experimental models of CNS injury [76].

AT-MSCs also have the potential to differentiate into vascular and myogenic lines. After injection into the vitreous cavity of a murine model for diabetic retinopathy (DR), these cells differentiated into pericytes located perivascularly, contributing to the preservation of the vascular architecture of the retina [77], perhaps suggesting a trophic and regulatory role for vascular retinal components. In vivo experiments show that AT-MSCs demonstrate morphological and physiological function of pericytes underlying the capillaries of adipose tissue [78]. AT-MSCs promote angiogenesis, improve ischemia-reperfusion, and have neuroprotective properties in experimental models of ischemic stroke [14, 78]. All these properties deserve further study in the field of vascular retinopathy (e.g., DR and venous and arterial occlusions) where AT-MSCs may be able to play a trophic role in recovery or protect against neuronal tissue damage.

Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

The highest density of progenitor cells in humans is in the bone marrow, where two main types of stem cell are present: hematopoietic stem cells (BM-HSCs; CD34+) and MSC or stromal stem cells (BM-MSCs; CD34-). The BM-HSCs give rise to all red and white blood cell lines, but the BM-MSCs have multipotent capacity to differentiate into many cell types of mesodermal origin, though with limited differentiation ability toward cellular elements of endodermal and ectodermal origin [79]. Both lineages can be isolated easily from bone marrow aspirates or plasmapheresis products. BM-MSCs specifically benefit form adhesive properties to plastic and the ease of identification by specific markers [73], as discussed in the previous section. BM-HSCs are usually selected by density gradient centrifugation or by immunomagnetic depletion and are positive for specific hematopoietic markers, such as CD34, CD14, CD45, CD31 (platelet-endothelial cell adhesion molecule), and glycophorin-A (erythrocytes) [79].

Although BM-MSCs constitute only 0.1% of the cell population of the bone marrow, they possess high in vitro proliferative growth and can be cultured and produced on a clinical scale. Similar to AT-MSCs, they show limited ability to dif-

ferentiate into neuronal cells, but they do possess the main properties of their secretome, which exerts a neuroprotective effect on the retina [80]. The neurotrophic secretome of BM-MSCs has been studied extensively and includes NGF, BDNF, NT-3, NT-4/5, ciliary neurotrophic factor (CNTF), GNDF, and PDGF [14]. Under hypoxic conditions, BM-MSCs increase the production and secretion of neurotrophic and angiogenic factors, such as CNTF, basic FGF (bFGF), and VEGF, in high concentrations [14, 70, 80].

An interesting feature of BM-MSCs, through interaction with cell surface proteins on endothelial cells, is their "homing" ability after intravenous administration. This homing phenomenon has been observed in experimental models where specific migration of BM-MSCs to tissues with inflammation has been observed. It is postulated that this cell targeting could occur in response to the expression of proinflammatory chemokines and adhesion molecules in damaged tissue [81, 82]. This property has aroused great interest for potential clinical application by intravenous administration, with the hope for neurotrophic and immunomodulatory benefits. BM-MSCs originally express nestin and other markers of glial origin and neural differentiation, such as GFAP and tubulin-βIII [83], evidencing a possible shared molecular pathway for neurogenesis.

Preclinical data demonstrate the therapeutic potential of BM-MSCs. In vitro differentiation studies have documented limited ability to differentiate into RPE and retinal neurons [14, 79]. After intravitreal administration of BM-MSCs in different experimental models of ischemia, retinal degeneration, and DR, cell migration with limited cellular integration has been verified in host retina with some differentiation toward RPE and PRs [84–86]. BM-MSC administration by subretinal, intravitreal, or intravenous injection has demonstrated neuroprotective effects on the evolution of retinal degeneration. Subretinal cell implants conferred a better protective effect on degenerated retinae [86, 87]. The neuroprotective effect of BM-MSCs has also been documented in animal models of glaucoma and optic nerve transection, where the prevention of apoptosis of retinal ganglion cells and the induction of axonal recovery have been observed [14].

BM-HSCs (CD34+) are widely used for the treatment of different hematological disease, and extensive knowledge has been accumulated courtesy through bone marrow transplants. Their homing ability is also well known, because CD34+ cells that are abundant in the bone marrow are mobilized to the peripheral blood either physiologically for reconstitution of the circulating blood or for recruitment in cases of vascular injury or tissue ischemia [79]. Although the underlying mechanisms of mobilization are unclear, it is believed that the release of angiogenic factors, such as the VEGF isoform A, may be one of the major pathways. Intravenous administration of autologous BM-HSCs has been used in different clinical trials, confirming the safety and efficacy of this approach in diseases with pathophysiological mechanisms related to tissue ischemia, including myocardial and cerebral ischemia [88, 89].

BM-HSCs mobilized into the peripheral blood give rise to a heterogeneous group of CD34+ cells with hematopoietic (e.g., white cells, red blood cells, and platelets) and endothelial (e.g., endothelial and perivascular cells) characteristics. As with BM-MSCs, therapeutic interest in BM-HSCs resides in the proangiogenic (e.g., VEGF, hepatocyte

growth factor [HGF], IGF-1, and FGF-2) and neurotrophic (e.g., CNTF, bFGF, and BDNF) properties of their secretome. In patients with diabetes, an increased number of circulating pro-inflammatory monocytes and a low number of circulating angiogenic cells (endothelial progenitors CD34+) have been found. Although the exact mechanism is unknown, it appears that the diabetic environment predisposes either to the inhibition of circulating angiogenic cells, to a defect in their homing mechanism, or even to their retention in the bone marrow [90]. This fact, recently corroborated by other research [91, 92], may underlie the characteristic of progressive vascular lesions in DR, which may occur because of the lack of a reactive physiological repair mechanism. Thus, the neuroprotective effect of BM-HSCs given by intravitreal injection remains of particular interest for the treatment of DR, and there is enough preclinical evidence to conclude that it has a reparative effect in the vascular system. Also, a rapid cellular homing phenomenon, targeted to damaged capillaries and vessels, has been demonstrated following intravitreal injection in murine models, both for DR and ischemia-reperfusion injury [93, 94]. The neurotrophic properties of BM-HSCs can also be combined with their capacity to repair the vascular system, through paracrine actions, once they are located around the damaged capillaries [95].

In murine models of retinal degeneration, intravitreal administration of BM-HSCs has shown a certain neuroprotective effect in preventing the progression of retinal lesions. The investigators considered a possible paracrine trophic effect because the implanted cells were aligned next to the retinal capillary network [96, 97]. Using a similar experimental approach, intravitreal administration of BM-HSCs in a murine model of hereditary retinal degeneration corroborated the phenomenon of preferential cellular homing beside the retinal vascular system, providing a neuroprotective effect mediated by the inhibition of retinal cell apoptosis [98].

The therapeutic potential of administering BM-HSCs intravenously is under discussion. Tissue and perivascular mobilization and recruitment have been demonstrated from the systemic circulation in response to certain cytokines that are secreted from the damaged neurosensory retina or RPE [94, 99, 100]. Some of these recruited cells even demonstrate a capacity to integrate into the subretinal space and to assume RPE-like phenotypes [101, 102]. In patients with nAMD, where the mechanisms of inflammation and angiogenesis are active in the retina, the number of circulating hematopoietic stem cells is increased. However, the clinical significance, as either a reactive mechanism for restoration/recuperation or a mechanism related to the choriocapillaris microcirculation, of this observation is unknown [103, 104].

The ability of BM-HSCs to differentiate into neural elements and RPE requires further exploration. The ability to differentiate into cells with RPE-like phenotypes has been analyzed in two BM-HSCs subpopulations (CD34+/CD38+ vs. CD34+/CD38-) that were cocultured for 7 days with human RPE cells. The results showed that the CD34+/CD38- subpopulation could acquire typical RPE morphology, express a series of specific markers (RPE65 and bestrophin), and acquire in vitro phagocytosis [105]. Given that these results imply functionality and some ability to differentiate into neuroectodermal elements, attractive possibilities are opened for their application as an alternative source of autologous cells in cell replacement therapies for RPE.

The available evidence indicates that BM-HSCs might be an excellent treatment option for rescue therapies in vascular, ischemic, and degenerative diseases of the retina and optic nerve. Indeed, despite their limited capacity for differentiation into neural and RPE lines, the paracrine neurotrophic properties and the associated homing phenomenon of BM-HSCs are attractive features. The number of clinical trials that have been carried out [35, 69, 79] or are currently underway reflects the perceived therapeutic potential of this cell line.

Umbilical Cord-Derived Stem Cells (UCSCs)

Blood obtained from the umbilical cord was first identified as a source of hematopoietic progenitor cells. Subsequently, cells with fibroblastic-like phenotypes have been isolated from Wharton jelly, consisting mainly of hyaluronic acid and chondroitin sulfate in a fibrillar matrix of collagen that protects the blood vessels—two umbilical arteries and one umbilical vein—of the cord. These cells were shown to be MSCs capable of adhesion to plastic culture dishes, which expressed CD29, CD44, CD51, CD73, and CD105, lacked CD34 and CD45 expression, and were able to differentiate into cells of adipogenic and osteogenic lineages in vitro [106]. Thus, they fulfilled the accepted international criteria for characterization as MSCs [107].

UCSCs have a very high capacity for in vitro differentiation to various cell lines, both ectodermal and mesodermal, as well as the ability to form neurospheres, cells of neuroglial origin (oligodendrocytes), and dopaminergic neurons [108]. Their immunomodulatory properties and exceptional ability to produce and secrete bioactive molecules that confer a paracrine mechanism for its therapeutic activity are also well known [109]. Therefore, based on the results of experimental models for neural and nonneuronal pathologies, their clinical applicability has gained increased relevance. Many clinical trials are now underway for their use in the treatment of different autoimmune and degenerative diseases [109, 110].

The secretome of UCSCs has characteristics that differ significantly from other MSCs. UCSCs show very reduced or even absent synthesis and secretion of the major proangiogenic factors (VEGF and angiogenin) and relatively increased production of some anti-angiogenic factors (thrombospondin and endostatin) compared to the other MSC lines. By contrast, they also express high levels of mRNA for other chemokines and proangiogenic growth factors not associated with classical VEGF-related pathways, with elevated secretion of neurotrophic factors including bFGF, NGF, NT-3, NT-4, and GDNF [111, 112].

Subretinal injection of UCSCs in a rodent model of retinal degeneration (i.e., RCS rats) preserves visual function and delays the degradation of PRs. In this classic experimental model, the neuroprotective action of UCSCs has also been used to rescue in vitro phagocytic dysfunction of RPE cells. The investigators postulated that the underlying mechanism may be related to the activation of the cell signaling pathways associated with the tyrosine kinase receptor ligands of BDNF, HGF, and GDNF [113]. Thus, UCSCs may be able to prevent or delay retinal degeneration through a rescue effect. This phenomenon may be clinically relevant as some patients with RP have a mutation identical to that occurring in RCS rats [114].

There is ample evidence to consider UCSCs for use as an alternative source of stem cells in degenerative pathologies of the retina and optic nerve, due to their proven plasticity, proven lack of tumorigenesis, and proven immunomodulatory properties [109, 110]. Likewise, the possibility of storing and preserving umbilical cords in biobanks, guaranteeing access to progenitor cells, and the number of registered clinical trials, which is currently growing, allows us to conclude that there is significant interest in the use of UCSCs for retinal therapy.

Induced Pluripotent Stem Cells (iPSCs)

Somatic cell reprogramming to produce induced pluripotent stem cells (iPSC) provides exciting possibilities for clinical application in various biomedical disciplines. Like ESCs, iPSCs are pluripotent stem cells with the ability to differentiate into any cell type, including RPE and PR cells.

Cell transfection with the forced expression of a combination of nuclear transcription factors—octamer-binding protein 3/4 (Oct3/4), Sox2, Krüppel-like factor 4 (Klf4), and c-Myc (OSKM factors)—in mouse fibroblasts has been shown to be sufficient to convert adult somatic cells into cells similar to ESCs [115]. One can reproduce this cellular reprogramming in adult human dermal fibroblasts [116, 117]. Although the underlying molecular mechanisms are still poorly understood, cell reprogramming can also be obtained by using combinations of other transcription factors, such as Lin28 and Nanog [118]. The reprogrammed cells then assume a state of undifferentiation, acquiring pluripotent and self-renewing abilities, and exhibit the potential to differentiate into different cells types, including tissues of endo-, meso-, and ectodermal origin. However, several problems inherent to the nature of iPSCs and the methodology used to obtain them need to be evaluated before one can judge whether the clinical use of iPSC-derived cells is safe. Key issues include the following: (1) they must not produce tumors or cancers; (2) they must retain their state of differentiation in situ; (3) they must not generate immunological responses from the use of viral vectors; and (4) they must remain located in the site of specific application of target regenerated tissue [119].

With the advent of different reprogramming techniques, direct administration methods have been introduced, e.g., the use of synthetic mRNA for the gene expression of cellular pluripotency. These represent significant advances in the induction of transcription factor expression or expression of molecules for cellular pluripotency that can replace viral-mediated delivery of transcription factors and possibly improve reprogramming efficiency. In this way, one may avoid using viral vectors or transcription factors that are potentially related to cell cycle control and oncogenesis [120]. Innovative strategies to replace integrative reprogramming techniques include the use of direct reprogramming protocols with modified synthetic mRNA (non-integrative episomal reprogramming), the use of selected transcription factors that do not induce tumorigenesis, and the optimization of clinical-grade xenofree protocols. This approach may facilitate clinical applications, allowing the control of the machinery of cellular synthesis through modulated and transient gene expression [121, 122].

Previous studies have shown that iPSCs can differentiate into several major cellular types of the neurosensory retina, including PRs and RPE cells [123–125]. Enrichment of cell culture media with retina differentiation-inducing proteins (e.g., Wnt, activin/nodal, and BMP) and the inhibitors related to their respective intracellular signaling pathways (e.g., DKK-1, lefty-A, and Noggin) can increase the efficiency of this differentiation. In addition, IGF-1, retinoic acid, activin, bFGF, nicotinamide, N2, and B27 neuronal supplements may contribute to better derivation of RPE [126].

By contrast, strategies used for the derivation of iPSCs toward PRs usually involve methodological changes, with it being necessary to obtain embryonic bodies maintained in cell suspension culture media for neural induction, which contain N2 and B27 supplements, as well as factors for inducing retinal differentiation, such as Noggin, lefty-A, DKK-1, and IGF-1. The cells are then seeded in high adhesion substrates for several months and allowed to develop. The addition of elements such as taurine, retinoic acid, and sonic hedgehog enhances differentiation toward PRs, because cells under these conditions are able to express the specific markers CRX, Nrl, opsin, rhodopsin, and recoverin [126].

Similar to ESCs, iPSCs cultured in vitro in 3D conditions can self-organize into structures with morphology similar to the neurosensory retina and can be adequately stratified with different retinal cellular elements. Furthermore, under 3D conditions, these cells may reach a very advanced degree of cell differentiation, which may lead to well-differentiated ganglion cells [127] and mature PRs, expressing specific molecular and cellular markers [128, 129].

There are several studies on the safety, efficacy, and functional integration of RPE and PRs derived from iPSCs implanted as a cellular suspension in the subretinal space in various murine and swine animal models [130–133]. Studies of the subretinal administration of RPE cell suspensions derived from iPSCs have demonstrated satisfactory host tolerance with a sufficient integration into the recipient tissue. These features have allowed some morphological and functional recovery of the PRs with some improvement in visual function, verified by electrophysiology [134, 135]. PR cells derived from subretinal iPSC implants have shown little migratory capability, though they can be grafted and express specific rod markers in the host outer nuclear layer of the retina after transplantation [130, 133, 136, 137].

Due to their ability to generate RPE and PRs from iPSCs, their host tolerance in preclinical studies, and the absence of ethical issues, unlike with hESCs, iPSCs represent an excellent potential alternative as an autologous cell source for treating retinal degenerative diseases. Also, their use for allogeneic transplants has been proposed, e.g., iPSCs from CD133+ cells in the umbilical cord blood of homozygotes for the HLA-A, HLA-B, and HLA-DRB1 antigens. For these antigens, human lymphocytes recognize RPE cells derived from iPSCs directly expressing HLA class I/II antigens. However, T cells failed to respond to HLA-A, -B, and -DRB1-matched iPSC-derived RPE cells from HLA homozygous donors [132, 138].

The first clinical trial using iPSC-derived RPE to treat patients with nAMD was started in 2014. The study was designed to assess the safety and tolerability of iPSC, and in the

long term, to establish its potential for prevention of progressive PR cell loss [1, 35]. RPE cells derived from iPSCs, obtained through integrative reprogramming, were transplanted into the subretinal space as "sheets" (1.3 × 3.0 mm) that spontaneously generated a cell monolayer without the need for artificial scaffolding [139].

The Posterior Segment of the Eye as a Target for Cell-Based Therapies

The retina not only detects photons, but it also processes visual stimuli using the well-defined substrate of second-order (bipolar cells) and third-order neurons (ganglion cells) as well as interneurons (horizontal and amacrine cells) [140]. The RPE phagocytose shed PR outer segments and provide essential molecular components of the visual cycle (e.g., 11-cis retinal) to PRs, a transfer of material facilitated through the interdigitation of PR outer segments with the apical microvilli of the RPE. There is thus a potential space between the PRs and the RPE that can be exploited surgically [68]. The BM is the substrate on which RPE survive and the subjacent choriocapillaris provides oxygen and nutrients (e.g., all-trans retinol) to the RPE and PRs. As mentioned earlier, PR precursor cells implanted in the subretinal space seem to improve retinal function in animal models although the mechanism (integration vs. fusion) is complex [141].

The posterior segment of the eye is easily accessible by intravitreal injection or current vitrectomy techniques. Also, the subretinal space can be surgically approached using improved instruments and specifically adapted cannulas to cause minimal damage to adjacent structures. The vitreous cavity and subretinal space, being small in size, require low volumes of a cell suspension or biomimetic carrier material to achieve good efficacy and biological responses [1, 12]. The relatively low numbers of allogeneic cells needed for a therapeutic benefit may be important in the case of allogeneic implants, as the low antigenic load may help reduce the likelihood of an adverse immune reaction [142]. Thus, transparent optical media facilitate not only access and visualization for procedures but also clinical follow-up after implantation.

The advent of novel noninvasive imaging techniques such as scanning ophthalmoscopy, optical coherence tomography, and adaptive optics tomography has allowed transplanted cells to be evaluated in a serial, real-time manner [4]. In addition, laser treatments are available to remove implanted cells in the event of abnormal or unwanted cellular behavior [1]. Electrophysiological tests for the retina (electroretinography and electrooculography) and optic nerve (visual evoked potential), as well as macular microperimetry, are well-standardized approaches that can objectively and serially evaluate the recovery of retinal functionality [42].

Finally, the eye is a highly compartmentalized organ characterized by relative immune privilege that makes it an excellent candidate for therapeutic cell-based approaches [143, 144]. It presents intraocular and subretinal microenvironments through the presence of internal (retinal capillaries) and external (RPE) blood–retinal

barriers. When performing a therapeutic procedure, the ability to use the contralateral eye as a response control can help with the clinical evaluation of therapy [10].

Pitfalls, Challenges, and Clinical Requirements

Significant advances have been made in cell-based therapies for the treatment of different degenerative diseases of the retina, which raises expectations for their translational potential. However, the clinical applicability of these results remains to be tested fully in humans. One of the important and as yet not fully resolved issues concerns ethical questions about the use of fetal and embryonic cells. Likewise, the diversification of regulations on the production, quality control, and use of progenitor cells in the context of advanced therapies requires standardization with a clearly established consensus [145–147].

More information is needed on the inherent risks associated with the potential of pluripotent cells to produce tumors and to induce an immune response in the recipient, as well as the current techniques of reprogramming by viral vectors [119]. The production of iPSCs through clinical-grade protocols in the absence of xenobiotics or through the process of match immuno-phenotypes are the focus of ongoing research [148, 149], as is establishing protocols for verification of the safety of the cell lines obtained [150]. Human T lymphocytes, for example, have the ability to directly recognize iPSC-derived RPE cells expressing class I/II HLA antigens, whereas they do not recognize RPE cells from homozygous donors for those antigens [132, 138]. Strategies that could replace integrative reprogramming techniques include the use of cell lines derived from iPSCs obtained through non-integrative (episomal) reprogramming protocols, the selection of transcription factors with direct reprogramming approaches, and the optimization of xenofree methods [151–153].

Another important concern is the issue of immune tolerance of allogeneic cells and genetically modified autologous cells [142, 154]. Theoretically, proteins and polypeptides synthesized de novo from the transcriptional processes of autologous cells modified by genes could provoke an immune response in the recipient tissue. Although it has been argued that ESCs may have some immune privilege because of their undifferentiated cellular characteristics [155, 156], it has also been shown that some terminally differentiated ESC-derived lineages can function as immunogens [157, 158]. Thus, there is a real possibility that long-term survival of allogeneic ocular transplants and possibly even autologous iPSC-derived transplants will require immune modulation.

Finally, it is important to emphasize that the clinical use of cell-based therapies in regenerative medicine requires the fulfillment of minimum requirements for clinical use in humans. In this regard, cell procurement and clinical-scale production, and the safety and therapeutic efficacy of the cell product must be checked and regulated. To this end, the European Medicines Agency (EMEA; http://www.ema.europa.eu/ema/) and the Food and Drug Administration (http://www.fda.gov/)

have extensive information and different projects to help make advanced therapies a reality.

Concluding Remarks

In recent years, great advances have been made in the use of cell-based therapies for the treatment of degenerative retinal disease. RPE cells derived from hESCs are being studied in phase III clinical trials of patients with GA and STGD. Although efficacy has not yet been established in these trials, there have been no significant safety signals thus far. However, the ethical issues posed by obtaining hESCs remain controversial and will require a broad consensus with clear regulations.

By contrast, BM-MSCs, AT-MSCs, and UCSCs may be effective for rescue therapies and are not constrained by these ethical issues. These cells show clear paracrine trophic properties with secretion of essential neurotrophic factors that allow their application in the earliest stages of degenerative processes in the sensory retina and RPE. In addition, MSCs demonstrate a homing phenomenon that may facilitate the application of cell suspensions by intravitreal as well as subretinal injections, with well-documented benefits on experimental ischemia-perfusion and DR models.

Novel cellular reprogramming techniques—whether integrative or episomal—represent the most interesting and promising advances in the field of cell therapy. However, some obstacles must be overcome before they can be widely applied clinically. Problems related to the potential for tumor formation, induction of tumorigenesis, and immunogenic reactivity are the main safety concerns. Adapting reliable clinical-grade approaches to reprogram cells into iPSC lines is another important issue for the maintenance of genomic integrity.

Once cell lines are established, they must be able to retain their pluripotency, allowing in vitro expansion and specific differentiation according to GMP standards. The possibility of generating allogeneic cells matched in homozygosis according to predominant histocompatibility antigens may obviate the need for immune modulation of allogeneic transplants in selected recipients. In the case of autologous cell transplantation in patients with hereditary retinal disease, gene correction of the iPSC before implantation will be required. Use of biocompatible substrates to support iPSCs may improve cell survival and physiological interaction with the host, and may facilitate their implantation. Similar to hESCs, iPSCs grown under 3D conditions can give rise to structures similar to a primitive optic vesicle and, subsequently, can generate adequately stratified organoids with the different elements of the neurosensory retina.

In conclusion, the preclinical and clinical results presented in this chapter reflect the significant advances made in recent times in the development of cell replacement/rescue therapies. Data from preclinical models indicate that these approaches may be useful for the treatment of degenerative diseases of the retina.

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Chapter 5
Manufacturing of Clinical Grade Cellular Products Under GMP Conditions

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Introduction

Diseases of the retina and optic nerve remain a major challenge in ophthalmic care, particularly the restoration of vision lost due to the death of neurons in these structures. Like other parts of the central nervous system (CNS), the retina and optic nerve have a severely restricted capacity for self-repair, such that damage to these structures now accounts for much of the untreatable blindness seen clinically. In particular, damage of this kind typically involves the photoreceptors or retinal ganglion cells (RGCs) and their axons. Nevertheless, considerable scientific progress has been made in this area, leading to the current wave of clinical trials in gene therapy and cell transplantation.

Early work in the area of neural tissue transplantation showed that immature retinal tissue could engraft in the rodent brain and give rise to normal retinal cell types, including photoreceptors and RGCs, as well as forming functional connections with visual centers in the host brainstem [1–3]. Other work explored cell transplantation as a method to replace retinal pigment epithelial (RPE) cells [4, 5], although that work appeared to rely on trophic effects more than cell replacement [6].

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Over the past two decades, workers have shown evidence of the remarkable integrative capacity of neural progenitor cells, including those derived from either the brain [7] or retina [8], as well as similar cell types derived from embryonic stem cells [9, 10]. More recently, work with more mature donor cells has raised questions about the possibility of subcellular exchange between donor and host photoreceptors and the extent to which this could account for apparent integration in such models [11]. Beyond academic considerations, this points to the potential use of donor cells as a means of providing trophic support via secreted factors or exosomes.

In fact, cells have also been transplanted to the human eye with the specific goal of rescuing host photoreceptors, particularly CNS progenitors from the brain and retina, as well as other cells types, such as those derived from the bone marrow [12–14]. Additional work has centered on the use of pluripotent cell-derived RPE [15–17]. These efforts have moved ahead relatively rapidly, given how novel they are, and at this time multiple programs using stem cells or cells derived from stem cells for treatment of retinal degenerations are now in clinical trials.

Integral to the use of such cells in humans has been an obligatory increase in attention to the manufacturing of allogeneic donor cells under Good Manufacturing Practice (GMP) conditions, as is required for general use in patient populations. Here we will focus on this particular topic and discuss key considerations and challenges facing the field.

Background

For many years, small molecule drugs, vaccines, and monoclonal antibodies have been the standard therapies for many diseases. However, over the past few years, cellular and gene therapies have started to show clinical efficacy and have been gaining more attention, as they are able to treat previously untreatable or incurable diseases, also in ophthalmic applications. Good Manufacturing Practice (GMP) has long been applied to the large-scale manufacturing of small molecules, vaccines, monoclonal antibodies, and proteins; for cellular and gene therapies, the initial product manufacturing runs were carried out mainly in academic centers, and efforts toward the application of GMP manufacturing have been limited to small cell numbers and lot sizes. Cellular and gene therapies, particularly chimeric antibody receptor (CAR)T cells, are now being used frequently for the treatment of leukemias and other malignancies [18] and are showing so much clinical success that it is anticipated that they will be entering the field of marketed cellular therapies under a biologics license from the US Food and Drug Administration (FDA) as soon as the late phase clinical trials are completed. Also CD34+ hematopoietic stem cell gene therapy for adenosine deaminase severe combined immunodeficiency (ADA SCID) is slated for marketing approval, since many children have had durable engraftment of gene-modified stem cells and correction of the disease [19]. Since these products are autologous cellular products and need to be manufactured on a case-by-case basis, technologies for GMP manufacturing developed in academic centers often

continue to apply and need to be adapted to commercial manufacturing of such cellular products. In contrast, if allogeneic cellular and gene therapy products will become successful and eventually commercialized, the need for reliable and reproducible large-scale manufacturing technologies of such products arises. The maintenance of clinical efficacy of large scale expanded, primary cellular products, under rigorous safety and efficacy standards is still a highly researched and greatly debated issue. GMP manufacturing of such products also remains time consuming and costly. We therefore want to discuss current approaches for the GMP manufacturing of such cellular and gene therapy products in the following paragraphs.

It is often thought that in the United States a GMP manufacturing facility needs a "GMP manufacturing license" from the FDA to manufacture cellular and gene therapy products for patient administration. However, this is only required for marketed products, but is not needed when these products are manufactured for clinical trials. The FDA will look at the GMP facility in the facilities description submitted in an Investigational New Drug (IND) application and will grant permission to manufacture such products under the IND if the facility is in compliance with rules and regulations for GMP facilities and GMP manufacturing set forth in Title 21 of the Code of Federal Regulations (CFR) Parts 210/211 [20, 21], providing general standards for GMP facilities and GMP manufacturing, and Parts 600/610 [22, 23], providing specific standards for biologics. Facilities and manufactured products are subject to inspection by the FDA, however, also if covered by an IND. Additionally, United States Pharmacopeia (USP) monographs also include recommendations for GMP facilities and product manufacturing [24]. It is important to point out that these chapters contain both required and recommended standards, the latter allowing for some flexibility, due to different facility layouts, allocation of personnel, and additional factors influencing facility operations.

It may be difficult to discern, from 21 CFR, what makes a manufacturing facility a GMP compliant facility. Lately, many people referring to GMP facilities and processes have added a "c" in front of GMP; however, the "c" only stands for "current" Good Manufacturing Practice, which means that the facility is operating according to "current" GMP rules and regulations; to operate under "current" rules and regulations is something a GMP facility should always do anyway.

In summary, the FDA and USP have been involved in regulating GMP manufacturing of biologics, in particular cell and gene therapy products, for quite some time and have put in place important guidelines that should be followed strictly, in order to manufacture a safe and potent product.

Personnel

The essence of a GMP manufacturing process is documentation. What comes to mind immediately is a written document outlining how a manufacturing process will be performed, step by step. This document is called a "Standard Operating Procedure" (SOP). Prior to the implementation of such an SOP, the manufacturing procedure has

to go through so-called "Engineering Runs," which allow for the SOP to be properly developed. Accurate documentation of all steps involved in manufacturing is an absolute necessity, in order to be able to perform a fully reproducible and controlled manufacturing process. The quality of the product, which pertains to product safety and efficacy, needs to be comparable from run to run.

It is therefore understandable that personnel involved in product manufacturing will also need to be regulated by SOPs. In the GMP setting it is required to have documentation for all individuals involved in a manufacturing procedure; in order to qualify such individuals, it is necessary that all personnel are trained and that this training is documented; no other personnel are permitted to manufacture the product. Specific training that needs to be conducted often includes how to gown, enter and exit the facility, work inside the clean room, how to handle specific products under aseptic conditions, and how to perform quality control. Training records must be generated for each applicable task and kept on file. Normally, all training is conducted by properly qualified GMP facility staff, for which the qualifications are also set in an SOP. Often, for a specific manufacturing setup, a new piece of equipment may be required. For personnel training, it may then be appropriate that an outside person, not part of the GMP facility staff, knowledgeable of this piece of equipment conducts the training; however, it must be fully documented and be in line with the requirements for training of GMP personnel. Training documentation is usually signed by both trainer and trainee [25, 26]. In addition, GMP personnel are to be familiar with essential skills not explicitly mentioned in product specific manufacturing SOPs, which includes proper handling of potentially biohazardous materials (also called "universal precautions").

There is also a defined reporting structure within the GMP facility; personnel report to the quality control (QC) unit, which controls and confirms that the manufacturing procedure was carried out as stated in the SOP [27]. In case a mistake was made during manufacturing, or a piece of equipment malfunctioned, it is recorded as a deviation and documented in a deviation report. This report is then followed up with appropriate corrective action, which is also documented.

Although personnel are supposed to follow SOPs without deviations during GMP manufacturing, GMP facility personnel are often well versed in troubleshooting and identifying needed process improvements. Under certain circumstances, particularly in cellular product manufacturing for Phase I and Phase II clinical trials, deviations from SOPs may be necessary to improve the process or product quality. During engineering runs it may not have become apparent, for instance, that certain process steps should be modified and improved, as only "real world" manufacturing conditions can reveal such challenges. These "beneficial" deviations are implemented into a new version of the manufacturing SOP, which can be used in later manufacturing runs.

A QC unit usually has, at a minimum, two staff members, which are designated as quality control (QC) and quality assurance (QA). It is important to recognize that their functions are independent from each other. QC directly oversees manufacturing processes and the personnel involved in manufacturing, generating documentation for these processes, while QA reviews the generated documentation and makes sure it is accurate [25]. Both QC and QA need to maintain high levels of integrity and be

able to properly identify problems and help with the implementation of corrective action. A word of caution: Problems in a manufacturing process can arise at any time, and it is vital to detect and correct them early so as not to compromise product safety or efficacy. Therefore, QC and QA have vital functions.

Large GMP facilities usually employ larger QC and QA units; however, small GMP facilities often struggle with personnel allocation to certain roles and functions. For this reason, there is some flexibility built into the regulatory structure; in smaller facilities, the functions of QC and QA have some latitude of interchangeability. For instance, QC and QA functions do not need to be performed by the same people at all times. It is possible that QC or QA personnel can be involved in manufacturing of a product, as long as there is proper QC and QA provided by other personnel, and the personnel performing QC and QA are properly trained and qualified, with the training and qualification being documented. However, one important rule always stands: QC and QA can never be performed by just one person at the same time, the separate functions of QC and QA need to be performed by separate persons.

Procedures

The GMP manufacturing of cellular products is often associated with larger scale cultures. While the overall tissue culture technique used is very similar to non-GMP technique, a much higher level of control must be applied to avoid contamination and associated safety issues when the product is administered into a patient. While cell cultures set up in a regular research lab could possibly have undetected contamination with mycoplasma, introduced by the operator and the environment, this would be completely unacceptable for a clinical grade product, as it poses a major safety risk for the patient. Therefore, the manufacturing process is highly regulated by Standard Operating Procedures and controlled by QC oversight.

An SOP for cellular manufacturing usually starts as a lab protocol, which is then transferred into the appropriate SOP format. Importantly, an SOP for the writing and formatting of an SOP is the basis for this step. Often it is the QC unit which helps the manufacturing group with drafting the specific manufacturing related SOP, determining if the manufacturing run is in compliance with GMP standards, and that important criteria such as safety, purity, identity, and potency of the product meet specifications. The SOP needs to list all materials and reagents required, and needs to describe, step by step, the manufacturing of a specific product. Also, all “in process” and “final release tests” need to be mentioned, as it is important to take, during and after manufacturing, appropriate product samples for these tests. The writing of such a procedure is not a simple undertaking; it often takes several revisions of the SOP to come to a document that actually works in the manufacturing environment and is easy to follow. Finally, the QC unit and the laboratory director sign off on the SOP, and the document is appropriately numbered and filed. Out of this SOP, a working document called a “Batch Record” is developed. This is a separate SOP that is used during the manufacturing run, into which all necessary entries, such as cell counts, volumes, and reagents added, are made during manufacturing.

It is the task of QC to oversee the manufacturing process of each product, and QC also assures that the manufacturing of the product fully adheres to the procedure described in the batch record, which is based on the manufacturing SOP [27–29]. QC verifies each step and then checks the step off in the batch record. This assures that product manufacturing is consistent and reproducible. Also, any materials used and samples taken are recorded in the batch record, and the proper application of labels to product containers is also recorded. It is often of great importance to properly time certain steps within the manufacturing procedure, and deviations from this timing should be avoided. Any culture procedure has enough inherent variables that cannot easily be managed; therefore, controllable external variables should be minimized. Only with proper timing and repeating the exact procedure, will the manufacturer be able to implement a path toward reproducibility in cellular product manufacturing.

After manufacturing is completed, the QC unit reviews the batch records and also the results from any tests performed in process or on the final product. It is common that such tests are performed in independent laboratories. These tests include viable cell counts (often Trypan Blue exclusion and manual hemocytometer count), 14-day sterility (testing for aerobic and anaerobic organisms and fungi), endotoxin, and mycoplasma [30–32]. Cell characterizations are also often conducted, which involve flow cytometry. Once the tests are completed, they are reviewed by the QA unit, and certificates of analysis are generated. The function of QA is to verify that the prescribed parameters for product quality have been met, which include safety, purity, and potency; QA is also responsible for the release of the final product [27]. It should be remarked, however, that in the United States for Phase I clinical trial products, a potency assay is not required and that the test procedures do not need to be validated but have to be properly qualified.

Materials and Reagents

Safety, purity, and potency of the materials used for GMP manufacturing must be known and must be within allowed specifications. In the US, the FDA will ask for materials and reagents used in the manufacturing process to be GMP grade or clinical grade whenever available. However, some products essential for the manufacturing of a specific product may not be available GMP grade. These products then need to be qualified for use by proper testing, and the certificates of analysis need to be presented to the FDA, who is the final authority to decide if these materials can be used. At the manufacturing facility, all materials and reagents need to be tracked, from the time when they arrive, during storage, and finally when used in the manufacturing process and eventual disposal. The QC unit is responsible for this task. All received materials are inspected for their physical integrity [33], that no damage occurred during shipping, and that the materials are really in correspondence with what was ordered. Reagents must also be received in containers with proper integrity and at the proper temperature. If shipped on dry ice, enough dry ice must be present for the product to

remain solidly frozen; items received refrigerated should have a temperature between 2 and 8 °C. For highly temperature sensitive reagents it is recommended that a datalogger will be included in the shipment that can record the temperature during shipping and report any deviation in temperature. All reagents also need to be checked for their expiration, and the expiration date must be within the time frame needed for product manufacturing. After initial inspection, materials and reagents can be labeled as accepted, rejected, or quarantined. Rejection can occur if damage was detected or if the shipping conditions were not met. In this case, the manufacturer or vendor needs to be contacted, and the materials or reagents might have to be returned. A quarantined condition of a reagent may occur if the certificate of analysis is missing, and the safety of the product cannot be determined; also, the product may still be in need of additional qualification testing not performed by the manufacturer or vendor. In this case, the product needs to be segregated until such testing is completed and the product has passed the required test specifications [34–36]. Once testing is completed, the QC unit reviews the results, and products can be released from quarantine after appropriate certificates of analysis for the tests are available. Appropriate storage areas for accepted materials and reagents are necessary, and complete traceability must be insured. Usually, for inventory control, the following information is required and needs to be recorded: Lot number, expiration date (if applicable), catalog number, and material description [37]. It should not be forgotten that lot number, material description, and expiration date of reagents used also need to be recorded in the product manufacturing batch record. This system assures that in the event of a product or manufacturing issue, materials and reagents are traceable to their origin, and the issue in question can be correlated with any materials and reagents used, if necessary.

Equipment

In order to be able to manufacture a cellular product under GMP conditions, several essential pieces of equipment, such as biosafety cabinets, incubators, refrigerators, freezers, and centrifuges, are required, for every manufacturing facility. These pieces of equipment, upon installation, need to be validated, so it can be assured that they conform to GMP standards. Equipment also needs to be properly monitored and maintained [38], so reliable and reproducible product manufacturing can be guaranteed, and equipment failure can be minimized or even completely avoided.

A distinction can be made between laboratory equipment and instruments. Large equipment or equipment of significant value (capital equipment) can be designated “equipment,” smaller pieces of equipment such as micropipettors can be designated “instruments.” Prior to use in GMP manufacturing, capital equipment should be validated. For any of these equipment validation procedures, three steps need to be considered: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) [39]. IQ deals with proper setup of the equipment, such as installation in the right area (e.g., proper ventilation, vents not obstructed) with proper electric current supply, proper leveling, proper assembly, etc. IQ is

performed according to a detailed checklist to ensure the equipment is installed appropriately, as intended by the manufacturer. OQ provides a determination of whether the equipment operates as intended, with all necessary equipment functions enabled. For instance, electric current uptake is normal, and the equipment operational values remain stable. This differs from PQ, which tells the evaluator if the performance of the equipment remains within certain set parameters, defined either by the manufacturer or by the end user. For instance, the temperature of a refrigerator should remain within set specifications within all parts of the interior. This may be ±1 °C within the interior, and is confirmed by temperature mapping. Also, after opening and closing the refrigerator door, the temperature should be coming back to the specified temperature within a specified period of time. After IQ, OQ, and PQ have been performed, a detailed validation report needs to be generated, which should be filed with the equipment records of the GMP facility. Yearly, the equipment needs to be retested and, if needed, recalibrated, to assure that the values recorded in the IQ, OQ, and PQ records are still maintained. Validation and retesting or recalibration are best performed by a specialized outside company, as it requires a good amount of technical equipment and knowledge, and also a conflict of interest can be avoided, if non-GMP personnel perform these procedures.

Particular attention should be paid to biosafety cabinets (BSCs). These are essential pieces of equipment for every cellular manufacturing facility, since they protect the cellular product from contamination from the outside environment and at the same time protect the user and the outside environment from biohazardous materials contained within the biosafety cabinet. It creates laminar airflow from top to bottom inside the cabinet that is maintained under negative pressure; the most prolific type of biosafety cabinet in cell manufacturing facilities is the recirculating type. Initially, the BSC needs to be validated at the manufacturer, then certified after installation in the manufacturing facility and recertified at least annually or semiannually, depending on the jurisdiction where the facility is located. The BSC itself acts as a small clean room with less than 100 particles greater than 0.45 μm per cubic foot per minute [40], which conforms to “Class 100” standards. If placed within a clean room this assures, with great confidence, aseptic product manufacturing, if proper sterile technique is applied. However, one caveat should be strongly underlined: the interior surfaces of a biosafety cabinet should never be considered sterile. The moment a tissue culture vessel or media bottle is placed within the biosafety cabinet, the work surface will become unsterile, if vessel or bottle is not sterile on the outside. Proper aseptic technique for product manufacturing is therefore essential [41].

Instruments, which are small pieces of equipment that do not require installation, usually do not undergo the abovementioned validation procedures of IQ, OQ, and PQ. They arrive either pre-calibrated from the manufacturer or can rather easily be sent out to calibration companies for calibration. Micropipettors or digital thermometers are good examples for this category. They do not require installation, as they are handheld. After the initial calibration, recalibration occurs either annually or semiannually. If there are a lot of instruments to be recalibrated, calibration companies also send technicians to the manufacturing facility to perform the

required recalibration on site. Interestingly, in some cases, already calibrated instruments may be used to calibrate other instruments. A calibrated thermometer, traceable to the National Institute of Standards and Technology (NIST), can be used to calibrate other thermometers within the manufacturing facility; however, an SOP for this calibration procedure needs to be written, strictly followed, and quality controlled.

Facilities

After controls for procedures, materials, reagents, and personnel have been established, the other most important aspect for the manufacturing of a safe and potent cellular product is the manufacturing environment. A GMP facility is a facility with a controlled environment with specific parameters for air cleanliness, temperature, and humidity, which need to conform to regulations set forth, in the United States, in 21 Code of Federal Regulations Parts 210 and 211 [20, 21]. The clean room environment for cellular manufacturing should be a Class 10,000 (ISO 7) environment, which specifies less than 10,000 particles greater than 0.45 μm per cubic foot per minute. A GMP facility for cellular manufacturing must minimize the risk of contaminants from the outside environment to reach the manufactured cellular product. Sturdy construction of such a facility is a requirement, using nonporous materials for walls and floors that do not release particles and can easily be cleaned [42], with seams between wall panels minimized. Ceilings in the manufacturing facility should be of similar quality, not releasing particles, and if possible, ceiling tiles should be avoided; gaps between ceiling tiles can allow fungus spores to penetrate and can also produce pressurization leaks. A solid ceiling may avoid these serious issues. A problem that is often overlooked is caused by light fixtures. Although modern lighting technology has made re-lamping less frequent, even light emitting diode (LED) lamps do malfunction and need replacing. Opening of the light fixture into the cleanroom environment causes a huge amount of contaminants, particularly fungus spores that were able to accumulate over a long time in the warm environment in the fixture to be released into the environment, which can seriously impact the cleanliness of the room. It is therefore recommended that light fixtures do not open into the clean room, but open into the space above or outside the clean room.

Air handling equipment should also be located outside the cleanroom, with easy access to it, so maintenance of the equipment can be performed without contaminating the clean environment [42]. The air handling system must be designed so all the air in the facility is either completely exhausted and replaced with fresh air (single pass air), or recirculated, with a specific amount of air being exhausted and replaced with the same amount of fresh air (recirculating system). It is also important to consider the appropriate number of air changes. A good number to aim for is 60 air changes per hour in manufacturing rooms. Before the air reaches the manufacturing rooms, it has to be high-efficiency particulate air (HEPA) filtered. A HEPA filter can filter out 99.999% of particulates under 0.15 μm [42, 43]. The most prudent way of

engineering such a HEPA filter system is to use a prefilter ahead of the HEPA filter, so the number of particles reaching the HEPA filter is lowered, and the life span of the HEPA filter is increased. Also, terminal HEPA filters in the ceiling of the cleanroom are advisable, since any particulates in the air duct that might arise over time due to duct aging or other mechanical problems will be held back by the terminal HEPA filter. If multiple HEPA filters are installed in the ceiling and properly spaced out and air exhaust vents are installed close to the clean room floor, laminar airflow can be achieved in such a manufacturing room. Although not required by the FDA, this strategy is highly advisable, since laminar airflow provides the best means of maintaining a clean environment. It is also important to properly plan the placement of large equipment such as BSCs, as they should be located in such a manner that laminar airflow is not obstructed [44].

GMP facilities for cellular product manufacturing should also be designed in such a way that air contaminants from the outside environment cannot enter the manufacturing rooms. This can be achieved by positively pressurizing the manufacturing rooms and providing a door interlock system. Positive air pressure in the manufacturing rooms toward anterooms will prevent any particulates from the anterooms to be carried into the manufacturing rooms. The door interlock system, which only allows one door to be opened at one time will prevent cross contamination with air from other manufacturing rooms through air turbulence. A word of caution, however, needs to be given: the manufacturing of aerosolizable products in positively pressurized manufacturing rooms needs to be avoided, since the positive air pressure will push aerosols into the anterooms, which can lead to all kinds of unwanted scenarios, including cross contamination issues.

In order to assure a controlled environment inside a GMP facility, regular quality control of the facility with its associated air handling equipment is required. An automated, electronic monitoring system that continuously monitors airflow, air pressure, temperature, and humidity and sends alarms to personnel in case set parameters are not met, is of great value. Essential equipment inside the GMP facility, such as freezers, refrigerators, and incubators, also need to be on this monitoring system. To be compliant with regulations, automated electronic monitoring systems need to have a 21CFR Part 11 compliant data storage and backup system [45]. In addition, it is recommended that automated monitoring is superseded by regular manual monitoring and generation of paper records for several important parameters: differential pressurization of manufacturing and anterooms by reading pressure gauges, room temperatures, refrigerator, freezer, and incubator temperatures by reading thermometers, and also CO_2 levels of incubators and humidity in manufacturing rooms using handheld equipment [42]. This is stressed so highly here since undetected equipment malfunction in spite of automated monitoring does occur, often caused by sensor or communication software problems. Daily manual monitoring and a quick response to equipment alarms can prevent catastrophic losses.

Another important aspect in a clean room environment for cellular manufacturing is viable and nonviable particle monitoring. When a clean room is initially certified, a thorough nonviable particle assessment is conducted, and the room is classified accordingly; annually or semiannually thereafter, an outside company should again conduct a

thorough nonviable particle count assessment for reclassification of the clean room. At the same time, HEPA filter function and integrity is checked, and air velocities in the room are also measured. Biosafety cabinets located in the manufacturing rooms should also be recertified at the same time. In order to provide assurance that during product manufacturing the particle count also remains within prescribed specifications, monitoring of nonviable particles within the room and also within the biosafety cabinet is advisable. This can be done with handheld devices, for instance, and the results can be recorded in a monitoring log. It is not anticipated that the nonviable particle counts change drastically between recertification periods; a drastic change, however, would require an investigation of the air handling equipment and HEPA filters. While nonviable particle counts provide good information about status and functionality of HEPA filters and the air handling equipment, viable particle counts are of vital importance for the safety of products manufactured in these clean rooms. Bacteria and fungus, particularly spores formed by these microorganisms, should never enter any cellular product, as serious consequences can arise if a contaminated product is administered into patients. Therefore, the air and surfaces in the manufacturing room need to be checked for the presence of viable particles; 10 cm trypticase soy agar plates are normally used for air sampling. These plates are either used as settling plates and exposed to room air for 3 h in certain predetermined areas of the manufacturing room or are placed into a device called "active air sampler" which draws, measures, and deposits a specified amount of room air onto the plate. Air sampling is also done inside a biosafety cabinet. After room air exposure, the plates are incubated for a week, and colonies are counted to determine the number of viable particles present. The limits for the number of colonies are specified in the USP [41]. To monitor the microbiological burden of surfaces, touch plates are used. These are smaller agar plates with a convex agar surface that allows the agar to touch room surfaces to be tested. Work surfaces inside the biosafety cabinets are tested, and, in addition, tables, door handles, and walls are sampled. The microbiological burden should be as low as possible; however, a clean room is not a sterile environment, but a clean environment. On the work surface of a biosafety cabinet, prior to the start of any work, no growth on a touch plate would be expected; however, surfaces outside the biosafety cabinet will, in all likelihood, yield growth of colonies, which is allowable. Again, the limits for the numbers of colonies allowed on touch plates are given in the USP [41].

To keep the microbiological burden as low as possible, it is necessary to perform regular cleanings of the facility. This includes cleaning of all surfaces, including walls and ceilings. In addition, great emphasis should be placed on frequently touched surfaces such as door handles, particularly on refrigerators, freezers, and incubators, and on table tops. Cleaning agents found to work well in cellular manufacturing facilities are cleaners containing quaternary ammonium and 70% ethanol. Cleaners may need to be changed if the microbiological burden increases over time. This, however, should be done in conjunction with an investigation of the microorganisms and their sensitivity to certain disinfectants [46].

One-way personnel, product, and waste flow in a cellular manufacturing facility is desirable, as it prevents backtracking and associated cross contamination of incoming products with outgoing products and vice versa. A good setup for such a one-way flow GMP facility starts with a gowning area followed by an intermediate room that can also

be used for incoming product storage, a manufacturing room, another intermediate room for outgoing product storage, followed by a de-gowning room. Pass-throughs allow one to move materials from the gowning room into the first anteroom, from there into the manufacturing room, from the manufacturing room into the second anteroom, and finally into the de-gowning room, according to one-way flow. Room doors and pass-throughs are interlocked, so only one door can be opened at one time, preventing cross contamination. Physical backtracking of personnel is prevented with this system, as personnel can remain in the manufacturing room and in the anterooms during manufacturing, passing-through materials, when the manufacturing procedure requires it. Product and waste also follows one-way flow in this system. It is acknowledged that not all GMP facilities have the luxury of one-way flow and need to apply the "clean corridor" system, where entering and exiting of the manufacturing rooms occurs from the same clean corridor. However, cross contamination issues can arise with this system, and it is necessary to implement strict spatial and temporal segregation for incoming and outgoing products and to regulate these procedures by good SOPs, with enforcement and control by QC.

Scale-up Methods

Over the recent years, cellular therapies, and also combined cellular and gene therapy products have shown increasing efficacy in treating and even curing diseases that were difficult to treat with conventional therapies or were even incurable. Very good examples are the treatment of ADA-deficient severe combined immunodeficiency with gene-modified autologous hematopoietic stem cells [19], treatment of certain hematologic malignancies with chimeric antigen receptor expressing T cells [18], and, most recently, treatment of retinitis pigmentosa with allogeneic retinal progenitor cells [47]. It is anticipated that the demand for cellular therapies will be strongly increasing in the next decade, upon FDA approval of such therapies as commercial, licensed products. This poses a huge question for manufacturers of such products: how can the anticipated large doses of these therapies be manufactured in an efficient and affordable way?

Currently, many cellular products for Phase I and II clinical trials are still manufactured in traditional flask cultures, an inherently open system, requiring highly trained user manipulation. The great advantage of this system is that that successful laboratory scale methods used for the development of the therapy can be immediately adapted for clinical grade manufacturing, and the cells can be visualized at every step. To a degree, this system can be scaled up by the use of larger flasks, multilayer flasks, and also multilayer cell stacks. Regular biosafety cabinets and CO_2 incubators can be used for the setup of these systems; however, the larger they get, the more cumbersome they are to handle. In addition, these open systems are contamination prone, the outcome is user dependent, and there are inconsistencies in gas perfusion and temperature distribution in certain areas of these large culture vessels. This is particularly apparent in 40 layer cell stacks. Although it can be envisioned that autologous cellular therapies may be manufactured successfully in such systems, it is not easily applicable for the thousands, if not millions of doses of allogeneic primary cell therapies needed in the future.

Pharmaceutical industry has already been using, for some time, large stirred tank bioreactors for the manufacturing of large doses of certain biologics. However, the products coming out of these large bioreactors were not primary cells, but predominantly products released by cells grown in these vessels. For many cellular therapies applying primary cells, it is vital to grow cells on surfaces, so they remain clinically efficacious; however, stirred tank bioreactors are not really designed to do that. Attempts have been made to adapt adherent cells to floating culture conditions or to grow cells on microcarriers that can be suspended in these bioreactors [48]. To date, these adaptation procedures are not entirely successful for manufacturing, large scale, a product that is comparable in all aspects to the cellular product grown in stationary culture on surfaces.

A different method that applies adherent growth conditions for cells and also employs a closed culture system is the hollow fiber bioreactor. The core of the system is a cartridge tightly packed with hollow polystyrene fibers; one of these cartridges allows for a cell growth surface area of more than 100,225 cm^2 flasks, or four 10-layer cell factories. The cartridge, at the same time, fits into one desktop culture control unit as small as a microwave oven. This unit is responsible for directing media flow through the cartridge, and at the same time controls temperature and gas tension. The plastic fibers are made of the same material as tissue culture flasks, and culture protocols for adherent primary cells can therefore be rather easily adapted to the hollow fiber culture system. Besides allowing for fluid perfusion through the center of the hollow fiber, it also allows for diffusion through the wall of the fiber, as it is porous. The pore size is small, however, and cells cannot enter or exit through the walls. The great advantage of the porous fiber walls is that an inner and outer fluid loop can be generated, and nutrients and metabolites can be replaced through the outer fluid loop and wall pores, while the inner loop can be maintained without fluid circulation. This is enormously helpful when more complicated cocultivation procedures need to be carried out. It can be shown that in this hollow fiber bioreactor system, primary cells grow in a very similar fashion as in tissue culture flasks, and fluid exchange is accomplished with very little shear force. The hollow fiber bioreactor system is a functionally closed system; cell seeding is accomplished from cell bags that are welded on to standard blood banking tubing using a sterile connecting device; medium is fed from media bags, also sterilely connected to the tubing. Cell harvesting is accomplished, as in culture flasks, by trypsinization followed by a washout. The cellular product is collected in a final product bag. One bioreactor cartridge can produce cells in the 10e9 range [49, 50]. Another very interesting aspect is that even gene therapy vector manufacturing can be successfully accomplished in this functionally closed system. This opens up the opportunity to produce gene therapy vector in laboratories that would normally not be equipped to handle gene therapy vector manufacturing.

For the future it could be envisioned that large numbers of closed system bioreactors will be “daisy chained” and controlled by a central control unit, producing the required cell doses for a large number of patients in a “nonclassified” space; a clean room environment is not needed, since the system is functionally closed; only the loading of the cell seeding bags and the unloading of the harvest bags and the final packaging/cryopreservation of the therapy would need to be performed inside a clean room. Such a system could be highly automated, and even a large manufacturing run could be handled by a small number of personnel. In addition, the manufacturing

is split into smaller sub-batches which can be pooled later on, if each batch passes quality control and conforms to set standards. This will guarantee successful large-scale batch manufacturing, even if a small sub-batch may fail, since many other sub-batches will most likely pass QC. Such a system would make it possible to adapt a current lab-scale protocol to a large-scale manufacturing process, while maintaining safety, and in all likelihood, efficacy in the human application.

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Chapter 6
Regulatory Requirements for Cell-Based Therapy for Degenerative Retinal Disease

Gary D. Novack

Introduction and Regulatory Environment for Cell-Based Therapy

Cell therapies present many unique challenges to regulators. Patients who are candidates for cell therapy are typically those with degenerative, debilitating diseases that are life- or sight-threatening. As a result of these conditions, the patients, or their parents, are at risk for abuse by unethical health providers who promote unproven therapies at excessive cash prices [1]. Cell therapy is also typically not covered by medical insurance—also putting patients at risk for financial abuse. Cell therapy is by definition a biologic product, an area of complexity and risk for adventitious organisms or genes, adding risks for patients that are not present with small molecule therapies. Testimonials of efficacy of these cell therapies by patients, including well-known public figures, continue to put pressure on quality science and clinical research. One report claims over 300 businesses in America are selling stem-cell therapies for a wide range of ailments [2, 3]. Concern over possible risks has been expressed by regulators and medical professional societies [4–7], and unfortunately some of these risks have been realized with poor clinical outcomes [8].

The European Medicines Agency (EMA) published a regulation in 2007 (http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2013/04/news_detail_001769.jsp&mid=WC0b01ac058004d5c1). They stated "…we are

This work has not been previously presented.

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aware that in some countries, the requirements for clinical studies of investigational cell therapy are not as rigorous as in the U.S. and Europe. This has led to patients with blinding or fatal diseases travelling to these countries for therapy. Such patients may be taken advantage of, and there are concerns." The EMA acknowledged the exciting possibilities provided by cell therapy for previously untreatable conditions. However, they state that like all treatments, these techniques also come with benefits and risks. These EMA regulations aim to ensure that medicines involving cell therapy are subject to appropriate authorization, supervision, and controls in order to reduce and manage those risks. The EMA makes a strong statement that permitting manufacturers to avoid compliance with quality standards, for example, by inappropriate reclassification of the treatment beyond the mandate of competent authorities for control of medicines, could risk exposing patients to cross-contamination and inadequate characterization of the cell preparations, resulting in short- and long-term risks for individual patients [9].

The Respiratory Cell Molecular Biology (RCMB) Stem Cell Working Group of the American Thoracic Society has a public statement on unproven stem cell interventions for respiratory diseases. They believe "…that the public and patients should have at their disposal, unbiased and scientifically sound information on therapeutic options including cell-based treatments." Further, they state that "…as with all medical interventions, patient safety must be the top priority of any prospective stem cell-based therapy or treatment. As yet, there is very little known about the short and long-term effects of administering any type of stem cell-based therapy to patients with lung diseases. Until we know more, we must be strongly concerned that the treatment could cause adverse effects and could worsen the patient's condition rather than improve it." They direct patients to these websites for registered clinical trials: the National Institutes of Health at www.clinicaltrials.gov and the EMA at https://www.clinicaltrialsregister.eu/ctr-search/search. The society is "…particularly wary of the ever-increasing examples of direct-to-consumer advertising of untested, unapproved, and potentially dangerous 'stem-cell' treatments that take place in several countries" (https://www.thoracic.org/members/assemblies/assemblies/rcmb/working-groups/stem-cell/).

At the time of the preparation of this chapter, no cell-based therapies are approved for the treatment of degenerative retinal disease. Thus, these are investigational therapies of unproven efficacy for which the benefit-risk ratio is not known. Among the regulatory issues are appropriate traceability of materials, treatment protocols, and patient follow-up measures, stressing protection of patients at the core of those rules. Simply stated, cell therapies must meet the same safety and efficacy rules as for all medicinal products, and the quality and manufacturing of these products as set out in Good Manufacturing Practice (GMP) requirements. Specifically, current Good Tissue Practice (cGTP) must be followed to prevent the introduction, transmission, or spread of communicable diseases by HCT/Ps (e.g., by ensuring that the HCT/Ps do not contain communicable disease agents, that they are not contaminated, and that they do not become contaminated during manufacturing; 21 CFR 1271.150(b), https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/tissue/ucm285223.pdf).

Development for Degenerative Retinal Diseases

This is a current issue in ophthalmology for the much simpler issue of autologous serum processing for the treatment of dry eye. In the U.S., enhanced regulation of compounding pharmacies (itself another social policy issue), including the use of current Good Manufacturing Practices, and a very short labeled shelf life (days, rather than weeks), have limited the availability of this product. In this context, blood sampling from a patient is subject to current regulations [10].

The potential for cell therapy begins with research described elsewhere in this book. Scientists evaluate cells of various origins, treated in various ways, for in vitro and in vivo activity that might be therapeutic or prophylactic in human disease. Once a possible treatment is selected, development starts.

Clinical use of an investigational therapy requires submission to a governmental regulatory agency (in most countries). In the U.S., this submission is called an IND (21 CFR 312.23). Regulatory agencies in other major countries have similar submission requirements. The IND includes three major areas of data: (1) the investigational product (also known as CMC), (2) nonclinical pharmacology, toxicology, and pharmacokinetics, and (3) the proposed clinical study and investigators [11].

Regulatory Guidances

Both the FDA and EMA have several guidelines on the use of cell therapies. FDA uses the phrase "human cells, tissues, and cellular and tissue-based products (HCT/Ps)" (FDA:http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm. November 2017; EMA: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003894.pdf, May 2008).

These guidances acknowledge that rapid scientific development has led to new, innovative investigational treatments with a high potential to treat previously unmet medical needs. The group of cell-based therapies "…are heterogeneous with regard to the origin and type of the cells and to the complexity of the product. Cells may be self-renewing stem cells, more committed progenitor cells or terminally differentiated cells exerting a specific defined physiological function. Cells may be of autologous or allogeneic origin. In addition, the cells may also be genetically modified. The cells may be used alone, associated with biomolecules or other chemical substances or combined with structural materials that alone might be classified as medical devices (combined advanced therapy medicinal products)." The complexity of cell-based products creates hurdles in their manufacture as well as the assessment of their safety in preclinical evaluation. Compliance with these procedures typically requires a commercial manufacturing facility that meets current GMP [12] and a preclinical facility that meets Good Laboratory Practice (GLP). Both guidances acknowledge that conventional nonclinical pharmacology and toxicology studies may not be appropriate

for cell-based medicinal products. Similarly, standard clinical development (e.g., Phase 1 studies in normal volunteers to determine maximally tolerated dose and pharmacokinetics; small, brief Phase 2 studies in patients to determine the magnitude of efficacy, dose-response, and duration of action; and then large, long-term Phase 3 studies to determine efficacy and safety) may not be appropriate. For some therapies, a single dose may essentially be a lifetime exposure. For others, pharmacokinetics (especially in the eye) may not be assessable. There tends to be a greater variability in the nature of the nonclinical and clinical program for cell therapy, albeit with a greater obligation on the developer to provide a scientific rationale and data to support the nonstandard development program.

All of the disciplines involved in product development are interconnected in an investigational product. For example, the nonclinical ocular toxicology conducted by toxicologists needs to be performed with drug product formulated by pharmaceutical chemists. Similarly, the drug product used in the clinical trials needs to be made according to GMP by manufacturing. However, this interaction between disciplines is much enhanced for cell therapy, as the drug product is a living system. Indeed, every "batch" requires not only chemical evaluation, but unlike small molecules, biological evaluation including identity (including long-term maintenance of differentiation as the desired cell type), purity, and potency.

Nonclinical Development

The FDA guidance on nonclinical assessment for cell-based products is organized in a manner akin to the standard triad of pharmacology (pharmacodynamic activity of the molecule both related to its desired effect, "on-target," as well as other activities, "off-target"), pharmacokinetics (how much drug or metabolite is in the eye or blood), and toxicology (untoward effects seen, typically at exaggerated ocular or systemic doses; Office of Cellular, Tissue and Gene Therapies (OCTGT) of the Center for Biologic Evaluation and Research (CBER) of the U.S. FDA (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM376521.pdf, November 2013). This guidance addresses not only HCT/Ps, but also the larger category of "…investigational cellular therapies, gene therapies, therapeutic vaccines, xenotransplantation, and certain biologic-device combination products" using the acronym "CGT."

However, there are differences. The guidance starts with an overall plan for the nonclinical program, including the biological plausibility of the therapy—which may come from the research studies noted previously. It then discusses identification of biologically active dose levels (i.e., pharmacology/ potency), establishment of feasibility and reasonable safety of the product by its intended route of administration (i.e., toxicology). The guideline then goes into intended clinical dose, patient inclusion/exclusion criteria, physiological parameters that can be used in the clinical study, and public health risks to administering this treatment. While these are similar to the development of any product, the description of these issues in early planning of nonclinical studies demonstrates the integrative nature of development of cell therapy.

The guidance then goes on to discuss the quality of investigational product used, species selection, and the standard toxicology testing. There is an emphasis here on the special nature of cell therapy. For example, the safety evaluation of human-derived cells in a nonhuman species may be inappropriate, and a species-specific treatment may need to be produced and tested (e.g., pig-derived rather than human-derived cells for preclinical experiments in pigs). Also, whereas the safety of small molecules is typically evaluated in wild-type rodents, rabbits, and larger animals, genetically modified rodents may be a more appropriate species to use if they exist.

With small molecules, adequate evaluation of *safety* (toxicology) in animals is required. Demonstration of in vivo on-target activity while *desired*, it is not *required* for regulatory allowance of an IND, although certainly some sense of potency for efficacy vis-à-vis potency for toxicity is instructive for developer and regulator [13, 14]. However, with cell therapy, demonstration of this activity is essentially required by the guidance. That said, animal models are not available for the full breadth of retinal degenerative diseases. Drug developers have tended to use Royal College of Surgeon (RCS) rats, which are a model of primary retinal pigment epithelium disease with secondary photoreceptor degeneration [15–18]. This model may be appropriate for some forms of retinal degeneration (e.g., Charbel Issa et al.) [19], but may not be as relevant for the more common retinal disorders such as geographic atrophy associated with age-related macular degeneration, although the RCS rat has been used in this context. In contrast, there may be better models for selected forms of autosomal recessive and autosomal dominant retinitis pigmentosa and for Stargardt disease [20, 21]. For toxicology studies, additional parameters specific to either the cell therapy product used and/or specific to the intended patient population may be required (e.g., humoral or cellular immune responses, putative biomarkers, and specialized histopathology).

Chemistry, Manufacturing, and Controls

From a manufacturing perspective, the preparation of cell therapy products requires extensive documentation of the many procedures and components involved including information on the components (such as detailed data concerning the derivation of the gene construct and characterization and source of cells), materials and procedures for manufacture of the product, the formulation (e.g., excipients) and container-closure system (e.g., bottle, syringe, etc.), product testing (including microbiological, sterility, fungi and adventitious agents, as well as identity, purity, contaminants and pyrogenicity), release criteria, stability testing, and storage requirements. For products other than autologous cells, scaling from a small batch to a larger product batch involves a number of procedures and testing to assure that the processes will produce the same product. Scaling up the manufacturing process of a stem cell-based therapy product can be an extraordinarily complicated process. If the product in question is for autologous use, the level of testing and donor issues are much reduced, but there is still the issue of using materials and procedures validated to provide potent, viable cells capable of safely and effectively producing the desired effect after collection, expansion, storage, transport, and delivery to the

implantation site. For cell lines intended to be used for more than one indication and not restricted for use in a single patient, the process is considerably more complicated, because an immortalized pluripotent cell line needs to be created, and a master cell bank established and characterized, as well as a working cell bank. Donor issues are greatly magnified, with higher legal standards and complexity initially. Even obtaining the source cells may require informed consents, an Institutional Review Board (IRB), requirements of the Health Insurance Portability and Privacy Act and the Office for Human Research Protection, and following a series of regulations on donor acceptability and testing from both the U.S. and European Union regulators. Once obtained and tested, a method of reprogramming the cells to produce induced pluripotent stem cells must be used that does not raise issues of undesired integration of viral vectors, by using methods that are efficient in terms of reprogramming from the original tissue to stem cells, which is not a simple task. Feeder cells and growth factors may be required to get the required yield, and they also must be characterized from reliable sources and meeting strict specifications. Then, the cell line cultures need to be expanded. Depending on the volume/ mass of the cellular product, production may move from single use processing materials to larger scale fermenters. Reproducibility of the process, between and within manufacturing campaigns needs to be demonstrated. Detailed characterization needs to be performed, as part of the process development, release testing and stability testing. These analytical tools need to be developed and validated during the drug development process, including morphologic characterization, use of phenotype-specific cell surface antigens, unique cell-specific molecular / biochemical markers, gene and protein expression analysis. Packaging and transportation of larger scale production products may also be more complicated, and shown to maintain the safety and efficacy of the product from production to use.

Clinical Development

A clinical development plan may be proposed when the information about manufacture of cGMP product for clinical use, and a nonclinical pharmacology and safety studies are available. As noted above, a typical clinical plan of using normal volunteers with small, single doses followed by higher and multiple doses in patients may be not applicable to cell therapies. The standards of Good Clinical Practice (GCP) apply, including a defined clinical protocol undertaken by properly trained clinicians and monitoring for compliance. In addition to submission of an IND, review and approval by IRB and written informed consent are required. For some therapies, only a single administration may be possible, and other types of therapy may be precluded. This possibility must be fully conveyed in a consent form. Cell therapies may require use of a Data Safety Monitoring Board (DSMB) [22] at an earlier stage than might be used for a small molecule. Some cell therapies require ocular and systemic immunosuppression, which is a nontrivial requirement, especially in older patients. Systemic adverse events possibly related to immunosuppression have been reported in cell therapy trials [23].

Clinical use of an investigational therapy requires approval from both an independent ethics committee (also known as an IRB), as well as submission to the federal regulatory agency in most countries. In the USA, this submission is called an IND (21 CFR 312.23). In this notification process, the Sponsor may proceed to clinical trials if FDA does not negatively respond within 30 calendar days following submission. Regulatory agencies in other major countries have similar submission requirements with regard to information required, although some are "approval" systems (i.e., an affirmative authorization is required prior to commencement of clinical trials).

Biological Licensing Application

When adequate information is obtained on the investigational product—clinical, nonclinical, and CMC, the Sponsor may apply for marketing approval by submitting a BLA, the biologics equivalent to a New Drug Application (NDA) for a small molecule.

Status of U.S. Regulatory Guidances

FDA's Center for Biologics Evaluation and Research (CBER) held 3 days of public meetings in September 2015 to obtain public comment on these draft guidances. The purpose of the meetings was to obtain comments on then existing draft guidance documents to "…provide clarity about FDA's existing regulatory framework for human cells, tissues, or cellular or tissue-based products (HCT/Ps)." HCT/P's are regulated as biologics.

Among the items discussed was whether an autologous transplant should be regulated. Some of these transplants are "same day" surgery in a patient, in which their own tissue is used. FDA issued a "minimal manipulation" exception ruling for such procedures in 2014 as follows: "…FDA's view is that autologous cells or tissues that are removed from an individual and implanted into the same individual without intervening processing steps beyond rinsing, cleansing, or sizing, or certain manufacturing steps, raise no additional risks of contamination and communicable disease transmission beyond that typically associated with surgery. FDA considers the same surgical procedure exception to be a narrow exception to regulation under 99 Part 1271." Some speakers at these meetings proposed a broadening of this rule. Procedures such as stem cell transplants for retinal disease involve obtaining a sample, processing it, and reinjecting into a different location. My understanding of FDA's *current* position is that all such procedures (which are currently considered investigational) require an IND with the narrow exception noted above. Of course, the phrase "minimal manipulation" may be subject to interpretation. Regulators tend to use a most conservative view, whereas investigators may choose a more liberal view. In my experience, it is safest to assume that the regulatory body will require an IND.

Some speakers referred to a bill pending in the 114th U.S. Congress, S.2689, the "Reliable and Effective Growth for Regenerative Health Options that Improve Wellness" or the REGROW Act. The proposed bill would amend the Public Health Service Act to require the FDA to conditionally approve certain cellular therapeutic products without initiation of large-scale clinical trials. A conditionally approved cellular therapy may be marketed if certain conditions are met, including conditions on the source, processing, and function of the cells in the product. This bill did not pass. As with any unapproved legislation, it may be resubmitted in the subsequent 115th U.S. Congress (2017-2018). The proceedings may be found as follows:

http://www.fda.gov/downloads/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/UCM530238.pdf
http://www.fda.gov/downloads/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/UCM532350.pdf
http://www.fda.gov/downloads/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/UCM532633.pdf

In August 2017, FDA Commissioner Scott Gottlieb, M.D. stated that while cell therapy has great promise in the field of regenerative medicine, it is "incumbent upon the FDA to make sure that this existing framework is properly defined, with bright lines separating new treatments that are medical products subject to the FDA's regulation from those therapies that are individualized by surgeons in such a way that they are not subject to FDA regulation" (https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm573443.htm). To that end, FDA conducted several enforcement actions to address a number of especially troubling products being marketed (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm573431.htm, https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm573427.htm).

Cited in one of the actions was the manipulation of the autologous cell transfer (as noted previously in this chapter). The detail is critical to understanding FDA's current position. FDA states that per 21 CFR parts 210.3, the firm uses "b4" components (drug products) which involve recovery of adipose tissue which is processed into a "stromal vascular fraction." FDA thus defines this product as three products: a drug, a biological product, and a human cell, tissue, or cellular or tissue-based product (HCT/P, 21 CFR 1271). The FDA concludes that the product does not "… meet all of the criteria in 21 CFR 1271.10(a), and therefore does not qualify for regulation solely under section 361 of the PHS Act [42 U.S.C. 264] and the regulations in 21 CFR Part 1271." Specifically, FDA states that the product "… does not meet the minimal manipulation criterion set forth in 21 CFR 1271.10(a)(1) and defined for structural tissue, such as adipose tissue, in 21 CFR 1271.3(f)(1)." FDA concludes that the product does not meet this potentially waiver as "…processing alters the original relevant characteristics of the adipose tissue relating to the tissue's utility for reconstruction, repair, or replacement." Thus, FDA states that since there is no BLA approval, it is illegal to market this product. Furthermore, even if considered investigational, there was no IND in place. Finally, FDA found "… evidence of significant deviations from current good manufacturing practice (CGMP) and current good tissue practice (CGTP)." Some of these deviations were found in a previous inspection in the fall of 2015.

Gottlieb stated that these two are "…examples of a larger pool of actors who claim that their unproven and unsafe products will address a serious disease, but instead put patients at significant risk." FDA plans to "…take additional actions in the coming months." So, while updated guidances are not available at the time of the preparation of this chapter (November 2017), FDA's position on cell therapy, at least where processing takes place, is more rigorous than some have thought, and is being made clear by these enforcement actions.

Based in part on the public meetings in 2015, the U.S. FDA recently issued updated guidances related to cell-based therapies that are applicable to treatment of retinal degeneration disease (https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm):

- Regulatory Considerations for Human Cell, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use; Guidance for Industry and Food and Drug Administration Staff.
- Same Surgical Procedure Exception under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception; Guidance for Industry.
- Evaluation of Devices Used with Regenerative Medicine Advanced Therapies; Draft Guidance for Industry.
- Expedited Programs for Regenerative Medicine Therapies for Serious Conditions; Draft Guidance for Industry.
- Deviation Reporting for Human Cells, Tissues, and Cellular and Tissue-Based Products Regulated Solely Under Section 361 of the Public Health Service Act and 21 CFR Part 1271; Guidance for Industry.

In these newly released guidances, FDA clarifies its view on same surgical procedure exceptions as: "…autologous cells or tissues that are removed from an individual and implanted into the same individual without intervening processing steps beyond rinsing, cleansing, sizing, or shaping, raise no additional risks of contamination and communicable disease transmission beyond that typically associated with surgery."

In conclusion, the potential benefits of cell therapy for patients with retinal diseases must be weighed against the risks of such therapy. These include not only the surgical procedures and sequelae, but also appropriate quality and controls on the cells used, and the manner in which they are handled during processing prior to administration to the patient. Worldwide, regulatory agencies are continuing to update their laws, regulations, and policies to reflect the changing science. These regulatory actions must balance the development and approval of novel therapies with protection of patients from undue risk and potential abuse from unethical organizations. Given the unique, variable, and changing nature of cell therapy, and the ongoing update of regulatory guidances, it is not possible to give a "one-size-fits-all" summary of the regulatory pathway for cell-based therapy for degenerative retinal disease.

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Chapter 7
Biomaterials and Scaffolds for Cell Replacement Therapy

Marta Stevanovic, Debbie Mitra, Dennis O. Clegg, and Mark S. Humayun

Introduction

Retinal degenerative diseases, which include retinitis pigmentosa and age-related macular degeneration (AMD), are leading causes of blindness worldwide. Retinitis pigmentosa is one of the main causes of progressive vision loss in younger individuals and affects approximately 1:4000 people in the world [1]. AMD is the primary cause of blindness in the elderly; it is predicted that AMD will affect 196 million people by the year 2020 and 288 million by the year 2040 [1–4]. Many retinal degenerative diseases are characterized by dysfunction of the retinal pigmented epithelium (RPE) cells, Bruch's membrane, choriocapillaris, or photoreceptors, which ultimately leads to photoreceptor death. A promising approach for treating such diseases is to derive healthy photoreceptors and/or RPE from stem cells and implant them into or under the diseased portion of the retina. In order to improve the

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function and integration of these transplanted retinal cells, various scaffolds can be used as supportive mechanisms. This review will address current developments in scaffold technology, specifically focusing on scaffolds that have been tested in vivo.

Stem Cell-Based Replacement Therapies

Photoreceptors and Retinal Pigmented Epithelium

The retina is a light-sensitive tissue that converts electromagnetic radiation into a neurochemical signal. Five major cells types comprise the retina, and vision depends highly on the spatial organization of these cells. The photoreceptors, or the cones and rods, are specialized light-sensing cells that convert light into an electrochemical signal, which is then relayed to the brain. Rods are responsible for scotopic, or low light, vision while cones process color vision [5–7].

The photoreceptors receive metabolic and functional support from the retinal pigmented epithelium (RPE), a monolayer of cells located in the subretinal space [8]. An important characteristic of RPE is the apical-basal polarity, a feature that facilitates bidirectional transport of nutrients, metabolites, water, and ions [8]. Tight junctions between RPE cells help form the blood-retinal barrier, which regulates the entry of many substances into the retina [8]. Other important functions of RPE include phagocytosis of shed photoreceptors outer segments, which is essential for photoreceptor survival; secretion of signaling factors; reduction of photo-oxidative stress by absorbing scattered light; and regeneration of 11-cis-retinal, an essential component of the visual cycle [8].

Stem Cell Sources

In recent years, strategies like regeneration, engineering, or replacement of cells have been investigated as viable treatments for retinal degenerative diseases. Human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) have become an attractive source for the derivation of photoreceptors or RPE. hESC can self-renew and are pluripotent, meaning they can differentiate into most, if not all, adult cell types in the human body. iPSC are created from mature adult cells that have been reprogrammed into an embryonic-like state. iPSC, like hESC, are pluripotent.

Both hESC and iPSC have been studied extensively as stem cell sources to treat retinal degenerative disease, but there are some challenges associated with their use. hESC-derived tissues are allogeneic and thus create a potential for transplant rejection due to a mismatch of surface major histocompatibility complex (MHC) proteins. Patients who receive MHC mismatched tissues require systemic

immunosuppression, which may be associated with negative side effects. Donor RPE with MHC proteins closely matching those of the recipient has been successfully implanted into primates without the use of immunosuppression [9]. These recipient primates, however, were healthy and had an intact blood-retinal barrier [9]. Eyes affected by AMD and other retinal degenerative diseases may have an impaired blood-retinal barrier, increasing the potential for immunologic rejection.

iPSC that are derived from a patient's own mature cells are perfectly MHC matched but may contain genetic mutations and damage from aging that would need to be corrected before they are implanted into the eye. Generating suitable autologous RPE from iPSC is a time-consuming and expensive endeavor. Furthermore, reprogramming protocols for iPSC may select for cells that proliferate rapidly [10–12]. These cells may have genetic abnormalities that increase tumorigenicity of the iPSC [10, 11].

Other than hESC and iPSC, there have been several other proposed stem cell sources. Undifferentiated bone marrow-derived stem cells and mesenchymal stem cells have been injected into animal models of retinal degeneration [13, 14]. Furthermore, gene editing has allowed for the creation of a novel stem cell line, the "universal stem cell" (Ucell) [15, 16]. Ucells do not express polymorphic MHC proteins on their surface, and thus dramatically reduce the risk of transplant rejection [16]. RPE has been successfully differentiated from Ucells in vitro [16]. Although these Ucells hold great promise, they have only recently been characterized and have yet to be tested in vivo as a treatment for retinal degenerative disease.

Photoreceptors Derived from hESC and iPSC

hESC have been differentiated into both retinal progenitor cells (RPC), which are precursors to photoreceptors, and photoreceptor-like cells [17, 18]. One challenge with differentiating stem cells into photoreceptors is contamination from other cell types [19]. While RPE can readily be differentiated and isolated from stem cells, the purification of photoreceptors can be difficult [19, 20]. Despite this challenge, several differentiation protocols have been developed [17, 21, 22]. For example, three-dimensional (3D) retinal culture has proven to be a viable approach to derive photoreceptors [19, 23–25]. These 3D-derived photoreceptors obtain elements of normal photoreceptor morphology, are responsive to light, and have characteristic electrophysiologic features [19, 23–25].

Lamba et al. 2009 were the first to transplant stem cell-derived photoreceptors in vivo [26]. Prior to transplantation, hESC cells were cultured in vitro with mouse retina explants; these cells differentiated into retinal progenitors, integrated into the retinal explants, and expressed photoreceptor markers [17]. When the hESC-derived retinal progenitor cells were transplanted into crx−/− mice, which are models of Leber Congenital Amaurosis, they further differentiated into photoreceptor-like cells and rescued light response [26]. Subsequently, several other groups have

studied stem cell-derived photoreceptors in vivo [20, 27, 28]. Photoreceptors and photoreceptor precursors derived from stem cells express photoreceptor markers, produce normal calcium oscillations, and rescue visual function [20, 27, 28]. When stem cell-derived retinal precursors were transplanted into mice with a complete deficiency of endogenous rods, formation of light-sensitive photoreceptors and rescue of visual function was observed [29].

Although it was previously thought that transplanted cells integrate with other retinal tissues via formation of neural synapses, recent discoveries have called this belief into question [30–32]. Findings that were previously interpreted as donor photoreceptor synapse formation may instead be due to exchange of materials from donor to recipient photoreceptors [30–32]. Even if transplanted photoreceptors do not form neural connections, however, the exchange of cytoplasm might still provide neurotrophic support to neighboring tissue [30–32].

Retinal Pigmented Epithelium Derived from hESC and iPSC

RPE has been the most studied retinal cell layer for therapy of degenerative disease. There are several methods for deriving RPE from stem cells, the most common of which is the spontaneous method [33–36]. Use of feeder-free and serum-free methods of differentiation, which allow for transplantation of RPE into humans, has been explored [22, 37, 38]. RPE cells can easily be isolated from other cell types during culturing because they are pigmented and have a unique cobblestone morphology. RPE differentiated from stem cells has been shown to obtain normal morphology and functional ability in vitro [39–42].

Cell Replacement Therapy as Suspensions

In vivo, stem cell-derived RPE is well tolerated and can rescue visual function. RPE cells can either be injected as a cell suspension or may be implanted as a monolayer supported on a scaffold. Several groups have demonstrated the survival of RPE cells injected into the subretinal space [40–43]. These cells stain positively for rhodopsin, indicating that they can phagocytose photoreceptor outer segments [39, 42]. They also integrate into the recipient's retina and rescue visual function [39, 40, 43]. A concern with injecting cell suspensions is that the cells may not localize to the areas where they are most needed and may also form clusters instead of adopting the desired monolayer formation [44]. Furthermore, during the process of injection, cells may reflux into the vitreous and result in severely damaging proliferative retinopathy [45]. One method of improving transplanted cell differentiation and survival is to rejuvenate the aged Bruch's membrane by treating it with bovine corneal endothelial cell extracellular matrix prior to RPE injection [46]. Enhancement of aged Bruch's membrane, however, has not been extensively studied. An alternative

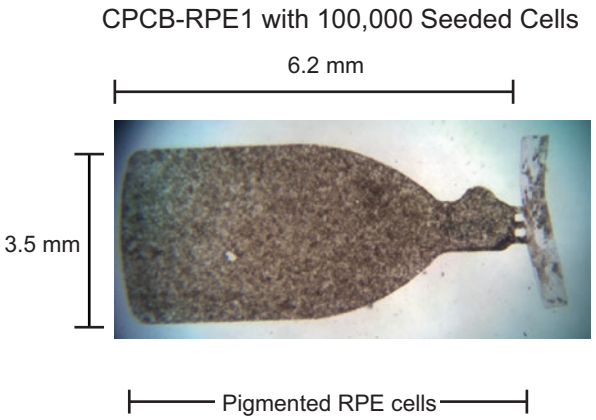

Fig. 7.1 Polarized monolayer of RPE cells seeded on CPCB-RPE1 parylene C membrane for clinical trial (NCT02590692). Approximate seeding density is 100,000 cells per membrane

to cell suspension is to implant RPE as a polarized monolayer, which may be more beneficial for rescue of visual function. When compared to unpolarized RPE, polarized RPE cells have an increased ability to phagocytose photoreceptor outer segments and are known to secrete a greater amount of pigment epithelial-derived growth factor, a protein that prevents angiogenesis and tumorigenesis and has neurotrophic properties [47]. Transplanted polarized RPE integrates well into the retina and rescues visual function [48]. In fact, monolayer transplantations have been shown to be superior to injections of cell suspensions in regard to RPE cell survival after transplant (Fig. 7.1) [49].

Bio-inspired Approach

Transplanting cells as a monolayer requires a supportive membrane that resembles the native Bruch's membrane. Bruch's membrane is a ~1–4 mm thick acellular pentalaminar structure comprised of an inner collagenous layer, elastin layer, outer collagenous layer, and basement membrane of choriocapillaris [50]. This semipermeable membrane, which lies between the RPE and the choroidal vessels, supports RPE on its basal side and regulates diffusion of materials between the retina and choroid [51]. Essential nutrients, electrolytes, and cytokines pass from the choroid, through the Bruch's membrane, and into the RPE while waste products from RPE pass through the Bruch's membrane to the choroid [52]. The integrity of this membrane is affected by aging, genetics, and the environment [51]. As the Bruch's membrane ages, it accumulates lipids, which calcify, and its hydraulic conductivity decreases [53, 54]. Both of these processes reduce permeability, inhibiting the transport of nutrients and waste [53, 54]. RPE seeded on deteriorated Bruch's membrane has low survival and develops abnormal morphology [55]. Since the native Bruch's membrane may be damaged due to aging and disease, an alternative structure is needed to support transplanted cells. Biomimetic scaffolds are a promising

substitute for damaged Bruch's membrane and have been shown to support formation, integration, and cell function.

Biomaterials and Scaffolds for Cell Replacement Therapy

Natural Biomaterials

Natural substances such as collagen, hyaluronic acid, gelatin, fibrinogen, silk, amniotic membrane, and anterior lens capsule have been considered as potential sources of scaffolds [45, 56–83]. An advantage of these materials is that they are biocompatible and closely resemble native Bruch's membrane [84]. Some concerns with using naturally occurring substrates are that they cannot be easily manufactured and thus may not be amenable to large-scale synthesis; the physical characteristics may be inconsistent, which can affect how the membranes behave in vivo; they have the potential to transmit disease; and they may be potential allergens [84–86]. Research on natural biomaterials is outlined below (Table 7.1).

Collagen Films *(Biodegradable)*

Collagen is a naturally occurring biodegradable substance that supports RPE growth and is biocompatible. ARPE-19 grown on type I collagen in vitro has normal morphology and phagocytic ability [56, 67]. In one study, even after the type I collagen scaffold was dissolved with collagenase in vitro, RPE cell sheets retained normal morphology, monolayer formation, expression of typical RPE markers, growth factor secretion, gene expression, and phagocytic ability [45]. The normal form and function of the RPE was attributed to the presence of laminin and type IV collagen, which were secreted by the RPE cells [45]. Laminin and type IV collagen create a basement-membrane-like structure that supports the RPE, even in the absence of type I collagen [45]. Retaining type I collagen for a short period of time in vitro allows the RPE to develop into a monolayer [45]. Dissolving it prior to implantation removes the exogenous collagen material, which has the potential to cause an immune reaction [45]. An important consideration before using collagenase is that the amount must be appropriately titrated so that enough is used to dissolve the type I collagen scaffold without causing damage to RPE cells (Figs. 7.2 and 7.3).

In vivo testing has shown collagen to be well tolerated immunologically and able to support RPE. When type I collagen scaffolds were transplanted into the subretinal space of rabbits, there was no evidence of inflammatory cell infiltration or fibrosis [56, 77]. Noncross-linked collagen has proven to be more suitable for supporting RPE in vivo when compared to cross-linked collagen. In one study, both noncross-linked and UV cross-linked collagen scaffolds seeded with human fetal RPE were inserted into the subretinal space of rabbits [77]. After the noncross-linked scaffolds

Table 7.1 Summary of natural membranes

Material	Biodegradability	Potential advantages	Potential disadvantages
Collagen [45, 56, 67, 77, 78]	Biodegradable	• Can be dissolved prior to implantation • Well tolerated in vivo	• If dissolved prior to implantation, the appropriate amount of collagenase must be determined. The cell sheet will not have any support in vivo • If not dissolved, may be too thick • Complicated surgical technique
Hyaluronic Acid [79, 80]	Biodegradable	• Biologically neutral	• Must determine optimal viscosity • May need to counteract effect of inhibitory retinal matrix proteins
Cross-linked Gelatin [57, 58, 81–83, 87]	Biodegradable	• Sterilization by gamma irradiation has proven viable in vitro and in vivo • Can be strengthened by cross-linking with EDC	• Transplantation into human subjects has not proven successful thus far • EDC at higher concentrations may be toxic
Cross-linked Fibrinogen [69, 70]	Biodegradable	• Microspheres support RPE in vitro and are well tolerated in vivo • 3D culture is beneficial for cells	• Not well studied
Silk [59, 60]	Biodegradable	• Bombyx mori silk fibroin is more permeable than Bruch's membrane • Antheraea pernyi silk fibroin is well tolerated in vivo	• Not well studied
Amniotic Membrane [71–76]	Nonbiodegradable	• Readily available • Has wound healing properties in the retina • Biocompatible	• Dispase, which removes epithelial cells, is toxic • Membrane is thick • Curling during surgery
Anterior Lens Capsule [61–66, 68]	Nonbiodegradable	• Similar exclusion limit to Bruch's membrane • If patient has cataracts, can harvest ALC during cataract removal	• Thicker than Bruch's membrane • Difficult to transfer cells to secondary tissue culture flask • Membrane folds • Difficult surgical procedure

dissolved, the RPE cells remained in a monolayer configuration, and the outer nuclear layer (ONL) and photoreceptors were preserved [77]. Cross-linked collagen scaffolds, on the other hand, did not dissolve, had poor RPE attachment, and caused degeneration of outer segments and ONL [77].

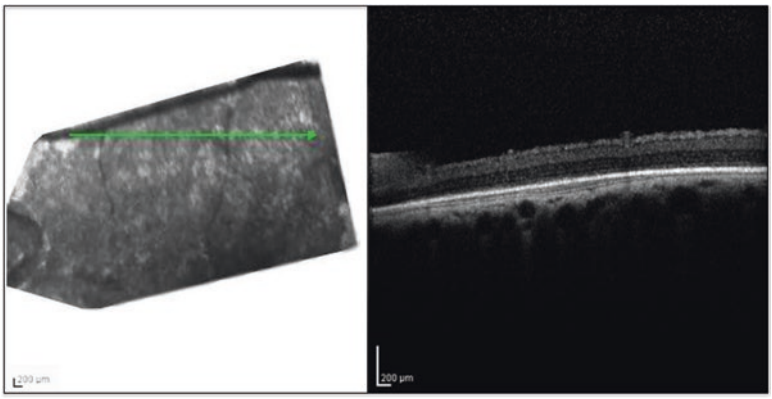

Fig. 7.2 CPCB-RPE1 parylene C membrane with seeded RPE implanted into the subretinal space of a Yucatan minipig

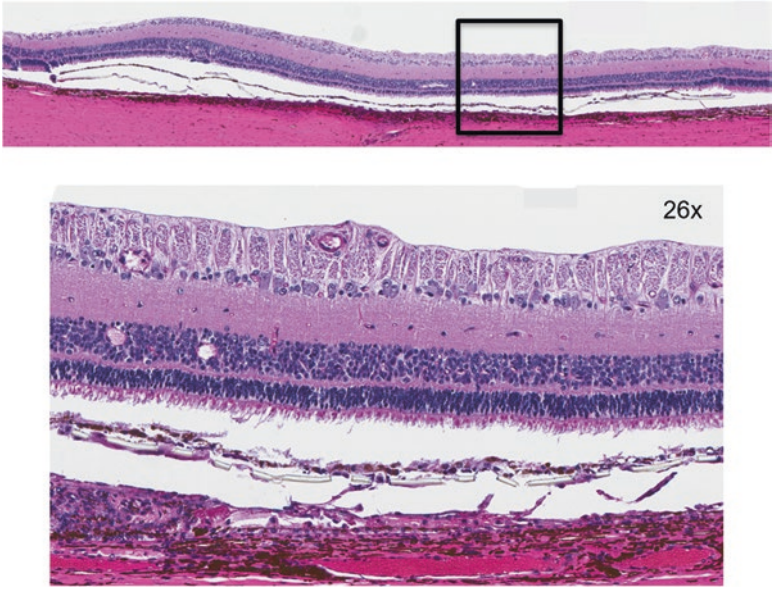

Fig. 7.3 Histology of CPCB-RPE1 membrane in subretinal space of a Yucatan minipig 3 months after implantation shows an intact monolayer of ESC-derived RPE with preserved photoreceptors

A clinical trial has shown the potential of using collagen membranes to treat wet AMD (JPRN-UMIN000011929). An autologous iPSC-RPE sheet was transplanted into one subject with neovascular AMD [78]. These RPE cells had been cultured on a collagen scaffold, which was dissolved with collagenase prior to implantation [78]. There were no serious adverse events during the 25-month follow-up, and the best-corrected visual acuity neither worsened nor improved [78]. Optical coherence tomography (OCT) revealed preservation of the retina adjacent to the graft [78]. In fact, some areas of the retina had a higher density, perhaps indicating recovery of photoreceptor inner and outer segments [78]. A second subject was enrolled in the study. However, transplantation was not performed because of concerns about DNA mutations that had been identified in this subject's iPSC-RPE [78]. Enrollment for the study was suspended in 2015 due to enactment of Japan's Regenerative Medicine Law, which requires regenerative medicine clinical research studies to be registered by medical institutions and not research institutions (Fig. 7.4) [78].

One challenge with using collagen is that, if not dissolved prior to implantation, it may be too thick and thus prevent necessary diffusion from occurring in the retina. When 7 μm thick type I collagen membranes without cells were implanted into the subretinal space of rabbits, there was degeneration of the retina in the transplanted region [56]. A thinner, 2.4 μm, membrane has been tested in vitro, but it is not known whether this membrane is stable enough to be used in vivo [67]. Another issue with collagen scaffolds is that the surgical technique required for implantation is complicated as is scaling up the product for clinical application [45].

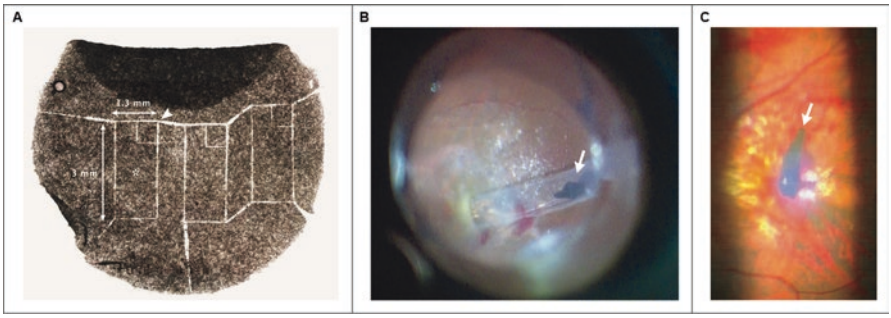

Fig. 7.4 Panel **a** shows the iPSC-RPE sheet used for transplantation. The graft, which was 1.3 × 3.0 mm in size, is denoted by an asterisk. Panel **b** shows the iPSC-RPE sheet (white arrow) that was transplanted under the fovea. Panel **c** shows the iPSC-RPE sheet (white arrow) 1 day post-transplant. Adopted with permission from Mandai M, Watanabe A, Kurimoto Y, et al. Autologous Induced Stem-Cell–Derived Retinal Cells for Macular Degeneration. *N Engl J Med*. 2017;376 [11]:1038–1046. doi:https://doi.org/10.1056/NEJMoa1608368

Hyaluronic Acid (HA)-Based Hydrogels *(Biodegradable)*

Hyaluronic acid is a naturally occurring biodegradable substance whose thickness, viscosity, and structure can be easily controlled when it is used in a gel form. HA plays an essential role in early development and is used as a feeder layer in stem cell culture [79]. HA gels are flexible and easy to inject into the retina. They are biologically neutral, as evidenced by the fact that subretinal injections of gels made from both HA alone and HA with methylcellulose do not disrupt retinal architecture [79, 80]. When RPCs encased in HA/methylcellulose gels were injected into the subretinal space of mice, they showed more even distribution, less aggregation, and better migration into the retina compared to RPCs injected in saline suspension [80].

An issue with using HA gels is that increasing the viscosity may decrease the RPC's ability to integrate into the retina. One study demonstrated that RPCs in cross-linkable HA gels do not show optimal integration with the outer nuclear layer when injected into rhodopsin −/− mice, which are models of retinal degeneration [79]. The lack of integration was attributed to two factors: the high viscosity of the HA that was used, which acted as a physical barrier to RPC migration, and the presence of inhibitory extracellular matrix proteins within the diseased retina, which prevented integration of the RPC [79]. Before HA is used clinically, an ideal viscosity would need to be determined for the human retina and the activity of inhibitory matrix proteins would need to be counteracted.

Cross-Linked Gelatin Scaffolds *(Biodegradable)*

Gelatin has shown promise as a scaffold in vitro and when implanted into animals. In vitro studies have focused on finding a method for sterilizing gelatin and on improving its strength. Gelatin sterilized with 16.6 kGy gamma irradiation is not cytotoxic to RPE in vitro, unlike gelatin membranes sterilized with hydrogen peroxide gas plasma or ethylene oxide [81]. When gamma-irradiated gelatin membranes seeded with RPE were transplanted into the subretinal space of rabbits, the implanted cells survived as a flat monolayer and did not induce an immune response during the 2-week follow-up [81]. RPE cells on gamma-irradiated scaffolds have also shown viability and tolerability in pigs. In one animal study, new basement membrane formation was observed 3 weeks after implantation of the RPE seeded on gamma-irradiated gelatin [82]. After 1 month, some regions had a monolayer of pigmented cells while others had multilayers with degenerated inner layers [82]. By this time point, the gelatin membranes had mostly dissolved [82]. The choroidal vessels and choriocapillaris, which depend on RPE for normal function, remained patent during the 3-month follow-up, and there was no evidence of inflammatory cell infiltration [82]. Transplanted RPE did survive for 3 months after surgery, but the number of surviving RPE cells was not quantified [82].

In addition to determining a suitable sterilization method for gelatin, studies have also focused on techniques to improve gelatin's strength. Because gelatin is known to rapidly dissolve in aqueous solutions, cross-linking can be performed to

stabilize and strengthen the gelatin scaffold [83]. Gelatin membranes treated with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) have been compared to those treated with glutaraldehyde (GTA) [83]. EDC cross-linked gelatin membranes were found to have minimal effect on rat iris pigment epithelial cell proliferation and expression of the inflammatory proteins IL-1β and TNF-α [83]. GTA-treated gelatins, on the other hand, decreased cell proliferation and increased IL-1β and TNF-α expression [83]. EDC- and GTA-treated scaffolds were then implanted into the anterior chamber of rabbits [83]. EDC-treated gelatin was well tolerated while GTA cross-linked gelatin caused persistent elevated IOP, corneal edema, and abnormal morphology of corneal endothelial cells [83]. Although EDC appears to be a suitable compound for cross-linking gelatin, EDC is known to be cytotoxic at higher concentrations [87]. If the EDC were to spread into the systemic circulation, it could have a deleterious effect on other tissues and organs [83].

Despite gelatin's encouraging results in vitro and in animal studies, it has not been quite as successful when tested in humans. In one case report, adult human RPE sheets from a cadaveric donor were placed into gelatin films and transplanted into a patient with wet AMD [57]. There was no improvement in vision during the 42 day follow-up [57]. The patient died of congestive heart failure 4 months after the transplant; subsequently, histopathology was performed and revealed multilayering of the RPE, attributed either to folding of the transplant or failure of transplanted RPE to attach to the diseased recipient Bruch's membrane [57]. In another study, 12 patients with wet AMD underwent transplant of donor RPE in gelatin sheets [58]. There was no improvement in vision, and several patients faced postsurgical complications, including cataract progression, retinal detachment, intraoperative retinal breaks, and macular pucker [58].

Cross-Linked Fibrinogen *(Biodegradable)*

Cross-linked fibrinogen spheres have been studied as carriers of human fetal RPE, demonstrating success both in vitro and in vivo. Freshly isolated human fetal RPE cultured in fibrinogen spheres can proliferate in cell culture for up to 3 months [69]. These spheres are easy to detach and transfer to a new cell culture well [69]. RPE grown through this system expresses cytokeratin, has microvilli, and demonstrates apical-basal polarity [69]. In vivo, one study showed that human fetal RPE cells grown in cross-linked fibrinogen microspheres were better tolerated in the subretinal space of rabbits than microspheres without any cells [70]. Thirty days after transplantation, histology revealed that human RPE had grown away from microspheres and formed a monolayer [70]. These eyes had less inflammatory infiltrate, photoreceptor loss, and choroid thickening than eyes that received microspheres without cells [70].

Silk *(Biodegradable)*

Membranes made from silk blended with other materials can support RPE in vitro and are biocompatible in vivo. *Bombyx mori* silk fibroin (BMSF) membranes are promising substrates, as they are four times more permeable than endogenous Bruch's membrane [59]. Vitronectin coating of BMSF has been shown to produce the best ARPE-19 attachment when compared to laminin, collagen IV, and fibronectin coatings [59]. When ARPE-19 cells were grown on porous BMSF membranes, they demonstrated a cobblestone morphology and tight junction formation after 8 weeks [59]. In vivo testing of silk membranes has focused on regenerated wild Antheraea pernyi silk fibroin (RWSF). When membranes made from RWSF combined with polycaprolactone, a synthetic material, and gelatin were transplanted subsclerally into chinchilla rabbits, there was no evidence of inflammation after 1 month [60].

Amniotic Membrane (AM) *(Nonbiodegradable)*

Amniotic membrane can be easily obtained post-cesarean section and has shown promise in vitro and in vivo as a support for RPE. While AMs with retained epithelial cells poorly support RPE growth, AMs denuded of endogenous epithelium help RPE obtain a normal morphology in vitro [73]. When rabbit RPE cells were cultured on human epithelium-free AMs, they developed the expected hexagonal morphology [73]. These cells also had greater pigmentation and apical-basal polarity than RPE grown on tissue culture plastic [73]. Furthermore, human RPE cells grown on epithelium-free AMs maintain their organization, epithelial morphology, and pigmentation [71, 72]. One concern with using denuded amniotic membranes is that dispase, a chemical used to remove epithelial cells, may damage the structure of the AM and may be harmful to any tissues it contacts [73]. Manual removal of epithelial cells is therefore preferable [73].

In addition to helping RPE develop normal morphology, AMs enhance RPE-specific gene expression. When compared to RPE grown on tissue culture plastic, RPE seeded on AMs demonstrates increased RPE65, CD68, and VEGF expression and comparable levels of CRALBP and tyrosinase [74]. RPE65 and CD68 indicate RPE differentiation while VEGF is important for retinal vessel development [74]. CRALBP is involved in the vision cycle, and tyrosinase is related to development of pigmentation in the RPE [74].

In vivo, AMs have shown wound healing properties in the retina as well as the ability to support functional iris pigment epithelial cells (IPE). In one study, epithelium-free porcine AMs were transplanted into Danish Landrace pigs with surgically removed RPE and mechanically damaged Bruch's membranes [75]. These AMs were able to reduce choroidal neovascularization and caused only minor inflammation [75]. Human AMs with Long-Evans rat IPE cells have been shown to rescue photoreceptors in Royal College of Surgeons rats, which are animal models for retinal degeneration [76]. These cell-membrane complexes do not induce immune cell infiltration for 12 weeks after surgery [76].

A major challenge with using AMs as carriers for retinal cells is that AMs are quite thick and may thus provide a barrier to nutrient flow. For example, the average thickness of transplanted AMs in one study was 32.12 μm, which is much greater than the thickness of native Bruch's membrane [76]. Before clinical use, AMs may need to be modified, either by thinning or through pore formation, in order to facilitate exchange of materials across the surface. Furthermore, AMs have a tendency to curl during the surgical procedure [75].

Anterior Lens Capsule (ALC) *(Nonbiodegradable)*

Anterior lens capsule is a natural component of the eye that has shown promise as a support for RPE in vitro and biocompatibility in vivo. Both ALC and native Bruch's membrane have comparable exclusion limits, which means that they can facilitate passage of similarly sized particles [61]. Pig RPE grown on autologous lens capsules obtains normal distribution of actin and stains for ZO-1, a tight-junction protein [62]. ARPE-19 cells cultured on porcine ALCs demonstrate increased ZO-1 and occludin expression, greater polarization, and enhanced transepithelial resistance when compared to ARPE-19 cells cultured on uncoated transwell polyester filters [63]. The effects of ALC on RPE phagocytosis function are unclear: one study reports increased phagocytosis ability for ARPE-19 cultured on porcine ALC while another demonstrates no difference in phagocytosis ability for ARPE-19 cultured on human ALC [63, 64]. Both studies used polyester membranes as a control [63, 64]. The observed differences in phagocytosis function may be due to cross-species binding specificity, autofluorescence of lens capsule, and effects of serum on cell activity [64].

Modifications to the seeding protocol can improve RPE function in vitro. Seeding by centrifugation produces RPE with greater metabolic activity and more epithelial-like morphology when compared to seeding by gravity alone [64]. Centrifugation changes the way cells bind to and distribute across the material, which ultimately leads to more homogenous and favorable cell characteristics [64]. Two other modifications to the seeding protocol are storing ALC membranes at −80 °C or exposing them to trypsin [65]. Both of these processes, which remove contaminating lens epithelial cells, have been shown to increase RPE density [65].

ALC scaffolds have shown biocompatibility in vivo; however, they are difficult to insert into the subretinal space. When porcine ALC membranes were transplanted into the subretinal space in pigs, there was no evidence of inflammatory infiltrate during the 2-week follow-up [66]. The surgical procedure, unfortunately, was fraught with difficulty due to curling of the ALC membrane [66]. In fact, ALC had such a great tendency to fold that the authors did not believe that flattening of the membrane by gas or perfluorocarbon liquids would be a sufficient improvement for clinical translation [66]. A second study that examined the feasibility of transplanting porcine ALC into the subretinal space of pigs corroborated these findings [68]. No inflammatory reaction was observed during a 6-week observation period in areas where there was no damage to the Bruch's membrane [68]. There was some photoreceptor loss, likely due to separation of the photoreceptors from the RPE

after insertion of the ALC scaffold [68]. Again, the surgery was difficult to perform due to folding of the membrane [68]. For some procedures, gelatin was used in an effort to flatten the ALC membranes [68]. However, gelatin was not sufficient to prevent folding, and it induced an inflammatory reaction in the subretinal space [68]. For some of the larger ALC scaffolds, the authors opted to use a DORC's spatula and forceps instead of a catheter [68]. The forceps did make the procedure easier to perform but required a larger retinotomy [68]. ALCs were also adherent to the forceps, making it more difficult to deliver the ALCs to the desired location [68].

The advantage of ALC membranes is that they can support RPE in vitro and are biocompatible in vivo. Furthermore, for patients who have both cataracts and retinal degeneration, autologous ALC can be harvested during cataract surgery and then used to support RPE to treat retinal disease [62]. Despite the advantages, a concern is that ALC is much thicker than Bruch's membrane, and thus may impede flux of nutrients and molecules [61]. One study also reported difficulty culturing RPE on ALC membranes in vitro; when the RPE cells were transferred to a secondary tissue culture flask, they failed to remain in a confluent monolayer [65]. In addition to these disadvantages, the difficulties associated with the surgical procedure would need to be addressed prior to clinical applications.

Other Natural Biomaterials

Other materials are being investigated as scaffolds in vitro such as basement membrane explant layers, cryoprecipitate, bacterial cellulose, and Descemet's membrane [88–92]. However, no additional in vivo data have been presented thus far.

Synthetic Membranes

Synthetic membranes are a viable alternative to natural materials as a source for scaffolds. The production of synthetic materials can be more easily controlled than that of natural substances, resulting in greater uniformity of the final product [85]. Synthetic scaffolds may be biodegradable or nonbiodegradable. Because biodegradable scaffolds eventually dissolve, they do not pose a long-term impediment to fluid and nutrient flow in the retina. However, degradation products from these scaffolds may have a deleterious effect on surrounding tissue [93]. Nonbiodegradable scaffolds, on the other hand, remain stable within the retina and help seeded cells retain normal morphology and function long-term, though they may obstruct transport of fluid and materials. Permeability of scaffolds may be enhanced through surface modifications, such as micropatterned holes. The size of the pores is critical in the design of porous membranes because they may have the potential to be blocked by cells [93]. An alternative approach that makes scaffolds more permeable and less damaging to surrounding tissue is to use nanowires, which are very thin [94]. Progress on the development of biodegradable and nonbiodegradable scaffolds is summarized below (Table 7.2).

Table 7.2 Summary of synthetic membranes

Material	Biodegradability	Potential advantages	Potential disadvantages
Thermally responsive biomaterials [96–99]	N/A	• Addition of EGF, ECM, and TGF- β2 can improve seeded cell function • No need to transplant these materials into the subretinal space	• Cells will lack support in vivo
PLLA and PLGA [100–117]	Biodegradable	• Well studied • Improve seeded cell function • Modifications like micropatterning, electrospinning, salt leaching, and culture as spheroids can further improve cell function • Adding laminin, MMP2, or small intestine submucosa improves function • Certain scaffolds promote RPC differentiation • Certain scaffolds have a low modulus, so are less likely to damage surrounding tissue • Well tolerated in vivo	• May be toxic • Certain scaffolds are brittle and thick, and may thus damage surrounding tissue
PGS [118, 119]	Biodegradable	• Promotes RPC maturation in vitro • Well tolerated in vivo • It has a lower Young's modulus than some PLLA/PLGA scaffolds, making it less damaging to surrounding tissue • Degrades more slowly than PLGA, so it less likely to negatively affect pH • Scroll injections, which reduce trauma, can be performed	• Not well studied
Polyurethane [120–122]	Biodegradable	• Supports RPE in vitro • Well tolerated in vivo	• Not well studied
PCL [94]	Biodegradable	• Supports RPC in vitro • Well tolerated in vivo	• Not well studied
PLCL [123]	Biodegradable	• Electrospun PLCL is well tolerated in vivo	• Not well studied
PET/Polyester [93, 123–125]	Nonbiodegradable	• Electrospun PET is well tolerated in vivo • Supports RPE in vitro • Short-term biocompatibility • Clinical trial indicated RPE survival for 12 months in two patients	• Requires pores

(continued)

Table 7.2 (continued)

Material	Biodegradability	Potential advantages	Potential disadvantages
PDMS [126]	Nonbiodegradable	• Supports RPE in vitro • O_2 plasma modification and laminin coating improve seeded RPE function and morphology. Modified membranes are well tolerated for 2 years in vivo	• Not well studied
Polyimide [127–129]	Nonbiodegradable	• Porous PI membranes support retinal cells • Laminin coating promotes RPE maturation • Biocompatible	• Difficult surgical procedure • Shooter instrument not useful due to PI flexibility
PMMA [130]	Nonbiodegradable	• Supports RPC in vivo for 4 weeks and is biocompatible	• Not well studied • Requires pores
Buckypaper [131]	Nonbiodegradable	• Supports RPE in vivo for 2 weeks and is biocompatible	• Not well studied
Parylene C [48, 132–136]	Nonbiodegradable	• Mesh improves stability of thin membranes • Tissue injector facilitates surgery • Biocompatible • Supports RPE in vivo • RPE on parylene C membranes rescues visual function • Ongoing clinical trial	• Requires pores

Thermally Responsive Biomaterials

Thermally responsive materials have been used during cell culture to facilitate the creation of RPE sheets. A major advantage of using these biomaterials is that they do not need to be directly transplanted into the retina, and thus cannot damage retinal tissue or induce an inflammatory reaction. Cells are cultured in vitro at 37 °C on these surfaces. When the temperature is reduced to 20 °C, the normally hydrophilic tissue culture surface becomes hydrophobic. Since cells do not readily bind to hydrophobic materials, they will detach in a monolayer formation.

Thermally responsive materials can support RPE in vitro and may even improve seeded RPE function. Type I bovine collagen decorated with linear chains of poly(*N*-isopropylacrylamide) is an example of a thermally responsive biomaterial [95]. When RPE cells were grown on this modified collagen surface, they remained viable [95]. RPE cells cultured on another thermally responsive material, poly(*N*-isopropylacrylamide-co-cinnamoylcarbamidemethylstyrene), demonstrated a similar ability to metabolize retinoid as freshly isolated RPE cells [96]. RPE cells that were grown on control tissue culture surfaces and dissociated enzymatically, on the other hand, formed a cell suspension and had poor retinoid metabolism [96].

Certain modifications to the thermally responsive tissue culture systems can further improve seeded cell function. Addition of epidermal growth factor (EGF) to thermally responsive copolymers of iV-isopropylacryl- amide, 4-(aminomethyl)styrene, and acrylic acid has been shown to improve seeded RPE cell proliferation [97]. Furthermore, including extracellular matrix (ECM) with the thermally responsive copolymers can enhance cell attachment [97]. Simultaneous grafting of EGF and ECM on the surface can improve polarized cell function [97]. Addition of TGF-β2 to culture media prevents damage to and shrinking of cells that usually occurs during detachment [98].

In vivo, RPE sheets grown on thermally responsive materials have shown encouraging results. When RPE cells were dissociated from poly(*N*-isopropylacrylamide) (PIPAAm) after 9–14 days of culturing, they retained a normal cobblestone morphology [99]. After transplantation into the subretinal space of immunosuppressed Dutch rabbits, the donor RPE attached to the host retina, including to the Bruch's membrane and the outer nuclear layer (ONL) [99]. There were some areas with nuclear notching and electron-dense cytoplasm, which are believed to be due to a mild immune response [99]. There was also wrinkling of the cell graft in certain regions [99].

Poly(L-Lactide) (PLLA) and Poly(Lactide-Co-Glycolide) (PLGA) *(Biodegradable)*

PLLA and PLGA scaffolds are able to support both RPE and RPC and, in some cases, improve seeded cell function. Six different scaffolds made from various blends of PLLA and PLGA (PLGA 75:25; high molecular weight (MW) PLGA 50:50; pure PLLA; PLGA 85:15; 10 micrometer thick PLGA from 50:50 lactic to glycolic copolymers; and 10 micrometer thick PLGA from 75:25 lactic to glycolic copolymers) were shown to support development of RPE with normal morphology in vitro [100–102]. Two of these scaffolds (10 micrometer thick PLGA from 50:50 lactic to glycolic copolymers and 10 micrometer thick PLGA from 75:25 lactic to glycolic copolymers) allowed RPE to develop normal tight junctions and to achieve a higher density than RPE grown on tissue culture plastic [101]. A seventh PLLA/PLGA scaffold, made from a 50/50 blend of PLLA 100k and PLGA 775, was shown to downregulate expression of immature RPC markers [103]. This finding suggests that the scaffold promotes differentiation [103]. When RPC seeded on this scaffold were transplanted into the subretinal space of rats, more than 50% of the cells survived for 14 days [103]. In addition to facilitating cell survival, this particular scaffold had a relatively low modulus and high elongation before failure [103]. A low modulus means that the scaffold is more compliant, and thus less likely to damage surrounding tissue.

Cell function in vitro can be enhanced by several physical modifications to the PLLA and PLGA scaffolds. These alterations include micropatterning, electrospinning, salt leaching, and creating spheroids. Micropatterned PLGA increases RPE attachment and improves morphology when compared to plain PLGA [104]. In addition, electrospinning of PLLA or PLGA nanofibers can be advantageous for

cell morphology and function. Randomly oriented electrospun nanofibrous PLLA scaffolds have been shown to induce conjunctiva mesenchymal stem cells to express photoreceptor-specific genes and to adopt a spindle-shaped morphology [105]. Furthermore, three-dimensional (3D) electrospun nanofibrous scaffolds made from 85:15 PLGA can improve RPE morphology in vitro [106]. When RPE cells were grown on these scaffolds, they developed normal tight junctions, performed phagocytosis, and expressed RPE65, a protein involved in the visual cycle [106]. They had a more natural intact monolayer and more microvilli than cells grown on two-dimensional (2D) PLGA scaffolds [106]. These results were not surprising given that 2D cell culture can negatively impact cell morphology, gene expression, and metabolism [107–110]. Furthermore, 2D scaffolds may be too dense and thus prevent diffusion while 3D nanofiber scaffolds provide a more permeable base [106]. Salt leaching of scaffolds to create pores is another viable alternative to using microspun nanofibers [111]. Porosity of scaffolds is a vital property, as it allows for diffusion of nutrients and other important compounds. Salt leaching allows for better control of pore size than does electrospinning and is a standard procedure to perform [111]. In addition to improving diffusion, salt-leached PLGA scaffolds have been shown to induce the retinal phenotype in mouse iPSC cells [111]. Another approach to improving RPE function is to culture RPE cells as spheroids in vitro. Spheroids are advantageous because they contain a high density of cells which can be easily stored and do not require exposure to enzymatic digestion in order to be transferred [112]. When human fetal RPE was grown on 50:50 PLA:PLG polymers in vitro, spheroid formation was observed within 48 h [112]. The RPE spheroids retained their differentiation, proliferative ability, morphology, and phagocytosis ability [112].

Adding coatings to PLLA and PLGA scaffolds can improve retinal cell function in vitro and in vivo. Laminin bound to PLLA facilitates attachment, growth, and maturation of RPE. When RPE cells were seeded on 70 nm PLLA scaffolds coated in laminin, they developed normal morphology, transepithelial resistance, phagocytic ability, and expression of RPE markers [113]. These membranes were well tolerated in the subretinal space of rdy rats; they did not cause retinal destruction, other than damage incurred during the surgical procedure [113]. Biocompatibility was assessed without the use of immunosuppressants [113]. There were whorls and rosettes seen on histology as well as microglial activation and decreased ONL thickness at 4 weeks [113]. These findings were attributed to retinal damage from the surgical procedure, which degraded the immune privilege of the eye, and not to the scaffold itself [113]. Another scaffold modification that has shown promise in vivo is addition of matrix metalloproteinase 2 (MMP2). RPCs that were combined with MMP2 PLGA microspheres demonstrated improved retinal integration and did not damage the retinal architecture when compared to RPCs transplanted without MMP2 [114]. The improved integration was likely caused by MMP2's inhibition of CD44 and neurocan, two molecules that are known to decrease integration of subretinal transplants [114]. The MMP2 was added to PLGA microspheres and not PLGA scaffolds in order to exclude any effects of a scaffold on RPC integration [114]. MMP2 is also beneficial when added to PLGA scaffolds. In

one study, RPCs seeded on a PLGA scaffolds with MMP2 were transplanted into the rho −/− mice, which are models for retinal degeneration [115]. The RPCs had appropriate photoreceptor morphology and expressed recoverin and rhodopsin, two markers for maturity [115]. In addition to MMP2, another beneficial modification to PLGA scaffolds may be to include small intestine submucosa. A hybrid scaffold containing PLGA and small intestine submucosa has been shown to improve RPE survival and phenotype expression when compared to a scaffold made from PLGA alone [116].

Although PLGA and PLLA have demonstrated positive results, there are still some concerns with biocompatibility and ocular toxicity [117]. Furthermore, both PLGA and PLLA can be brittle and thick, which increases the risk of injury after insertion and may decrease diffusion [100, 115].

Poly(Glycerol Sebacate) (PGS) *(Biodegradable)*

Although not studied as extensively as PLLA and PLGA, PGS has shown promise as a carrier for RPC and may be advantageous when compared to PLGA and PLLA. When RPC were seeded on a PGS scaffold, they developed mature phenotypes in vitro based on analysis of mRNA, protein expression, and glutamate sensitivity [118]. After transplantation into the subretinal space of mice, the RPCs migrated into the recipient's retinal tissue and the PGS membrane completely dissolved 30 days after implantation [118]. The RPCs expressed markers indicating neural cell type (NeuN) with the desired photoreceptor (crx, rhodopsin) and bipolar (PKC) fates [118].

PGS has several advantages when compared to PLLA and PGLA. It has a Young's modulus that is fivefold lower than that of scaffolds made from 50/50 PLLA 100k/PLGA 775 [119]. A lower Young's modulus indicates that it is a more elastic material and thus less likely to damage surrounding tissue. PGS also degrades more slowly than PLGA and is therefore less likely to negatively affect the pH of its environment [118, 119]. Furthermore, scroll injections, which reduce surgical trauma, can be performed using PGS [118].

Polyurethane *(Biodegradable)*

Polyurethane can support RPE cells in vitro and has shown compatibility in vivo. One study examined the feasibility of using polyurethane membranes created from different starting materials. Two types of membranes were used: PUD5 and PUD6 [120]. PUD5 contains a soft segment derived from poly (capro-lactone) (PCL) while PUD6 has a soft segment created from both poly(ethylene glycol) (PEG) and PCL [120]. Both PCL and PEG have nontoxic degradation products [121]. The PUD5 and PUD6 membranes were able to support RPE attachment in vitro [120]. When the two membranes were implanted into the subretinal or suprachoroidal spaces of Brown Norway rats in vivo, there was preservation of retinal architecture,

intact RPE above the implant, and no evidence of inflammation for 15 days after surgery [120]. Polyurethane membranes derived from montmorillonite clay have shown similar results when implanted into rats [122].

Polycaprolactone (PCL) *(Biodegradable)*

PCL nanowires can support mouse RPCs in vitro and in vivo. When mouse RPCs were cultured on laminin-coated novel nanowire PCL scaffolds, they demonstrated cell adhesion, proliferation, and expression of mature bipolar and photoreceptor markers [94]. The PCL scaffolds with RPCs were then transplanted into rho−/− mice [94]. In vivo, the RPCs integrated into the retina and differentiated morphologically after 1 month [94].

Poly Lactide Caprolactone (PLCL) (Biodegradable)

One study has shown that PLCL can support RPE. PLCL, a biodegradable material, was compared to poly(ethylene) terephthalate (PET), which is biostable [123]. These two different substances were chosen in order to elucidate the effects of topography on cell function that is independent from the type of material used [123]. Even though this study did not focus on the substances themselves, it still provides important information about how these materials behave in vitro and in vivo. 200-nm-thick electrospun scaffolds made from fibers of PLCL or PET were able to support RPE in vivo [123]. These scaffolds were then transplanted into the subretinal space of rabbits. There was variable migration of native RPE, reactive gliosis, and PR degeneration above the membrane [123]. The gliosis was attributed to the surgical procedure, which damages parts of the retina while the photoreceptor loss was likely due to insertion of artificial barrier that impedes movement of nutrients [123].

Poly(ethylene Terephthalate) (PET)/ Polyester (Nonbiodegradable)

PET membranes, commonly referred to as polyester, have shown promising results both in vitro and in vivo. Adult human RPE grown on polyester membranes demonstrates normal polygonal morphology, presence of tight junctions, expression of RPE genes, and presence of visual cycle proteins RPE65 and CRALBP in the cytoplasm [124, 125]. Polyester membranes tested in vivo have shown short-term biocompatibility. In one study, 10 thick porous polyester membranes were inserted into the subretinal space of rabbits [93]. Histology performed 14 days after surgery showed no evidence of inflammatory cell infiltration or retinal toxicity [93]. There was subretinal scarring and ONL atrophy, which was attributed to disruption of the retina during the surgical procedure [93]. Endogenous RPE cells were able to migrate over the polyester implant, but they did not produce a monolayer [93].

Polyester membranes seeded with RPE have also been transplanted into the subretinal space of rabbits [124]. In the rabbit retina, atrophy over the implant was observed 1 week after surgery [124]. This degeneration, which subsequently stabilized, was attributed to the scaffold inhibiting diffusion of nutrients [124]. Four weeks after surgery, the RPE survived as a continuous polarized monolayer [124]. Another study examined the use of polyester membranes in pigs [125]. hESC-RPE on human-vitronectin-coated polyester membranes survived in the subretinal space of pigs for up to 6 weeks posttransplant [125]. At this time, rescue of photoreceptors was seen in the eyes implanted with hESC-RPE on polyester scaffolds while there was no photoreceptor rescue observed in control eyes implanted with only polyester scaffolds [125].

A Phase I clinical trial has shown promising results (NCT01691261) [125]. hESC-RPE cells grown on porous polyester scaffolds were implanted into the subretinal space of two human subjects with wet AMD [125]. The transplanted hESC-RPE survived for 12 months after surgery [125]. The presence of patchy autofluorescence over the scaffold suggested that the hESC-RPE was performing phagocytosis [125]. ERG testing revealed decreased photoreceptor function in both subjects at the 6-month follow-up, which improved in one subject at the 12-month follow-up but persisted in the other [125]. Both subjects demonstrated visual fixation over the patch, increased reading speed, and improved contrast sensitivity during the study [125]. Best-corrected visual acuity improved 29 and 21 letters, respectively [125].

Polydimethylsiloxane (PDMS) ***(Nonbiodegradable)***

One study of PDMS showed its ability to support RPE in vitro as well as its long-term stability in vivo. PDMS was modified with O_2 plasma and coated in laminin [126]. O_2 treatment increased the hydrophilicity and granularity of the surface while laminin was added to improve cell adherence, morphology, and survival [126]. In vitro, the modified PDMS scaffold (PDMS-PmL) had better RPE attachment, proliferation, differentiation, maturation, tight junction formation, PEDF secretion, pigmentation, and phagocytic ability than the unmodified PDMS scaffold control [126]. The modified PDMS scaffold was also able to support a multilayer of tissue that consisted of RPE and photoreceptor-precursor cells [126]. After in vitro testing, both the modified PDMS-PmL scaffold and the unmodified PDMS scaffold were implanted into pigs [126]. The unmodified PDMS scaffold caused loss of host photoreceptors and RPE and retinal detachment in some animals [126]. The PDMS-PmL implant had more encouraging results. The photoreceptor and endogenous RPE layer remained intact, and there was complete retinal attachment around the implant [126]. There were no inflammatory cells and the retinal vasculature was preserved [126]. Multifocal electroretinography (ERG) testing 2 years after implantation revealed preserved macular function in the eye with the PDMS-PmL scaffold but not in the one with the PDMS scaffold [126]. Trophic levels of PEDF were maintained in the eyes with PDMS-PmL implants [126].

Polyimide (PI) ***(Nonbiodegradable)***

Although PI membranes have shown positive results in vitro and have demonstrated biocompatibility in vivo, using PI membranes clinically may be limited because they are difficult to manipulate during surgery. Retinal cell cultures grown on porous PI membranes have exhibited survival in vitro [127]. Coating PI in laminin promotes hESC-RPE maturation and growth as pigmented monolayers [128]. Studies using organotypic cultures of rat retina have shown that porous PI membranes are sufficient for diffusion of nutrients [127].

In vivo, subcutaneous implantation of PI membranes in mice demonstrated the biocompatibility of PI [127]. The inflammatory response that developed after implantation subsided after 6 days [127]. After 4 weeks, there was minimal scarring and no fibrous capsule around the implant [127]. When hESC-RPE on ultrathin porous PI membranes was placed into the subretinal space of immunosuppressed rabbits, there was a decrease in pigmentation, likely due to loss of hESC-RPE, and presence of inflammatory cell infiltrate and retinal atrophy [129]. These findings are possibly attributed to insufficient immunosuppression and damage to hESC-RPE and retinal tissue from surgery [129]. hESC-RPE damage may be caused by exposure of PI membranes during surgery. Although use of a shooter instrument to insert the cells and membrane can protect the seeded RPE from harm, PI membranes are too flexible to be compatible with such a device [129].

Poly(Methyl Methacrylate) (PMMA) ***(Nonbiodegradable)***

One study examined RPCs grown on either smooth or porous laminin-coated PMMA scaffolds, which were subsequently placed into the subretinal space of C57BL/6 mice [130]. Four weeks after implantation, porous scaffolds enhanced RPC attachment, facilitated cell migration, and improved cell integration when compared to smooth scaffolds [130]. No inflammatory cell infiltrate was seen [130].

Buckypaper ***(Nonbiodegradable)***

Buckypaper is formed from aggregates of carbon nanotubes. It is a nonbiodegradable substance that has been investigated as a scaffold. When human RPE cells cultured on buckypaper were transplanted into the subretinal space of rabbits, there was normal RPE morphology and the buckypaper remained flat 2 weeks after surgery [131]. There was no evidence of inflammation or edema [131].

Parylene C ***(Nonbiodegradable)***

Parylene C is a nonbiodegradable substrate that has been used successfully in vitro, in animal studies, and in clinical trials. One study examined the effects of several coatings on polyimide membranes and found parylene to be one of the superior

coating materials. Coated and uncoated polyimide membranes were implanted into the subretinal space of Yucatan pigs [132]. Twelve weeks after implantation, all animals had ERG amplitudes at or above the baseline, and histology revealed a loss of photoreceptors and disorganization of the ONL and inner nuclear layer (INL) in all animals [132]. Animals implanted with the parylene-coated membranes had no loss of cells in the INL while animals implanted with amorphous aluminum oxide-coated (AAO), amorphous carbon-coated (AC), and uncoated membranes experienced loss of INL cells [132]. Overall, parylene caused less retinal disruption on histology than AAO-coated, AC-coated, and uncoated membranes [132]. Animals implanted with parylene did not have a statistically different outcome in retinal anatomy when compared to animals without any implant [132].

In addition to being well tolerated in vivo, parylene C can mimic Bruch's membrane and support RPE in vitro [133]. Parylene C membranes that are 0.15 μm and 0.3 μm thin have similar permeability to healthy human Bruch's membrane [133]. A concern with making ultrathin membranes is that they may not be able to withstand manipulation during surgery [133]. A solution to improving the strength of the membranes is to support them on a mesh frame. Mesh-supported submicron parylene C membranes (MSPM) have been shown to sustain development of RPE with normal morphology, tight junctions, microvilli, and polarization [133].

The CPCB-RPE1 scaffold is a MSPM that is tolerated well in vivo. When implanted into the subretinal space of Yucatan minipigs, CPCB-RPE1 parylene C membranes seeded with hESC-RPE caused minimal change in the retinal architecture and no inflammatory cell infiltrate after 1 month [134]. The transplanted hESC-RPE survived and histology revealed possible interdigitation between the host photoreceptor outer segments and apical sides of the CPCB-RPE1 [134]. Some retinal thinning was observed [134]. Because both the implanted animals and control animals had retinal thinning, this finding was attributed to laser treatment and not to toxicity of the parylene C scaffold [134]. RPE seeded on parylene C membranes is not only well tolerated in vivo, but it can also rescue visual function in rats. One study examined the effect of vitronectin-coated rCPCB parylene C membranes on RCS rats [48]. Some rats received membranes seeded with hESC-RPE (rCPCB-RPE1) while others received membranes coated in vitronectin (rMSPM + VN) [48]. As in the Yucatan minipigs, there was no major inflammation or damage to retinal architecture after implantation with the membranes [48]. There was a focal ONL preservation in both the rCPCB-RPE1 and rMSPM-VN groups [48]. Survival of hESC-RPE was demonstrated in 87% of animals who received rCPCB-RPE1 [48]. In addition to safety and survival, functionality was also assessed. The rats implanted with rCPCB-RPE1 had better rescue of rods and response to light on superior colliculus testing than those with rMSPM-VN [48]. Both implantation groups had improved visual acuity [48].

Although these ultrathin parylene membranes have shown promise to treat retinal disease, surgically implanting them into the subretinal space can be challenging because of their thinness and fragility. The required surgical procedure can be technically complex and may cause damage to surrounding tissue. A novel tissue injector for implantation of hESC-RPE on CPCB-RPE1 (MPSM) membranes has been tested during membrane implantation in Yucatan minipigs [135]. Use of the injector reduced surgical complications, improved hESC-RPE cell survival, and decreased tissue trauma [135].

The CPCB-RPE membranes are being tested for treatment of dry age-related macular degeneration in a Phase I/IIa clinical trial, which is funded by the California Institute for Regenerative Medicine and sponsored by Regenerative Patch Technologies, LLC) (NCT02590692). The primary objective of the study is to test the safety and tolerability of subretinal CPCB-RPE1 implantation in patients with geographic atrophy with involvement of the central fovea. The secondary objective is to test changes in visual acuity, visual field, and retinal function. Adverse events from implantation are also being monitored. Preliminary reports from this study have yielded encouraging results [136]. Five subjects were enrolled and followed for time periods ranging from 120 to 365 days. OCT imaging demonstrated integration of the transplanted hESC-RPE with the host photoreceptor layer. During the study, the average number of fixation events increased and two out of five subjects showed improvement of fixation from "unstable" to "stable" over the implant area [136]. Four out of five subjects had no worsening of visual acuity and one subject demonstrated a 17 letter improvement in acuity [136].

Conclusion

The future of retinal cell transplant rests on the development of an ideal biomimetic scaffold. Restoring the architecture of the retina requires transplanted cells to be in a monolayer formation on a thin, permeable membrane. Cells seeded on scaffolds show improved survival and function when compared to cells injected as a suspension. Many different natural and synthetic scaffolds that are biodegradable and non-biodegradable are being studied as potential carriers of retinal cells. Several scaffolds tested in vivo show encouraging results, and a few have even been used in clinical trials. In addition to determining the ideal scaffold, it is important to explore the use of surgical tools and techniques to aid in the implantation of biomimetic scaffolds and seeded cells. Further research in these fields will enable clinicians to more successfully treat retinal degenerative diseases.

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Chapter 8
Transplantation Immunology: Retinal Cell-Based Therapy

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Ocular Immune Privilege

The success of retinal transplantation in the subretinal space is contingent on many factors including the recognition that ocular immune privilege exists in this site. In 1948, Medawar [1], motivated by the work of HSN Greene in 1940, observed that genetically incompatible tumor cells would grow when transplanted into the anterior chamber of the eye or brain but not when implanted subcutaneously. He interpreted this unexpected growth potential as failure of the immune system to reject allogeneic grafts and coined the term "immune privilege." Thus, immune privilege is defined as those host sites where foreign tissue grafts can survive for extended periods of time while similar grafts placed in conventional sites are acutely rejected. It was subsequently recognized that certain tissues, e.g., the cornea, are immunogenic but have the ability to protect themselves from destruction by the host immune response [2] and thus, exhibit "immune protection."

Controversy surrounded the immunologically privileged status of the eye until inbred strains of rats were used to definitively demonstrate its existence within the anterior chamber using skin grafts transplanted across both major (MHC) and minor (mHC) histocompatibility barriers [3]. Several factors were found to restrict immune privilege—the magnitude of immunogenetic disparity between donor and host, graft size, and type of tissue grafted. It was suggested that the unique properties of the anterior chamber relied upon the forced intravascular presentation of antigen and consequent aberrant central processing.

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In 1970, Kaplan and Streilein [4, 5] made the surprising observation that allogeneic lymphoid cells injected into the anterior chamber of normal rat eyes induced a deviant form of systemic immunity. Rather than being ignored by the host immune system, the original concept of immune privilege, the alloantigens on injected lymphoid cells induced a robust antigen-specific antibody response. Moreover, the recipient rats had an impaired ability to reject orthotopic skin allografts genetically identical to the transplanted cells. The term "immune deviation" was used to describe this phenomenon. Subsequent studies by Niederkorn et al. [6–9] and Streilein et al. [10] indicated that the immune deviation induced by the anterior chamber inoculation of antigen was not a function of the injected lymphoid cells but a characteristic of the anterior chamber. They coined the term "anterior chamber-associated immune deviation (ACAID)" to characterize this phenomenon [11]. Subsequent studies demonstrated that a wide range of antigens including soluble proteins, particulate antigens (e.g., viral particles), histocompatibility antigens, and tumor antigens could induce ACAID. Furthermore, immune privilege also exists in the vitreous cavity and subretinal space but is abrogated when the retinal pigment epithelium (RPE) in the eye is destroyed prior to transplantation [12, 13]. The immunological hallmarks of ACAID are dependent on an intact spleen and include the generation of primed effector CD8+ T cells, B cells that produce IgG non-complement-fixing antibodies, and inhibition of CD4+ Th1 T cells (i.e., delayed type hypersensitivity) [4, 5, 10, 14, 15].

The biologic importance of immune privilege in the eye is apparent in the host's immune response to infectious pathogens. For example, an antigen-specific immune response in the liver might effectively eliminate a pathogen and in the process destroy adjacent tissue by the associated leukocyte inflammation (i.e., the bystander effect) without irreparable damage to that organ because of its size. In contrast, such bystander tissue injury in the eye would have a devastating effect on vision if the fovea of the retina was inadvertently destroyed causing loss of central vision.

Both anatomic and functional factors contribute to the development of immune privilege in the eye including the blood-retina barrier, absence of lymphatic drainage, soluble immunomodulatory factors, immunomodulatory ligands on the surface of ocular parenchymal cells, chronic activation of the complement system, and tolerogenic antigen presenting cells (APCs) [16–25]. The development of immune privilege is complex and involves many different regulatory mechanisms, the details of which are too numerous to discuss in this chapter and have recently been reviewed by others [26, 27]. Instead we will focus on the three major strategies used by the eye to modify the innate and adaptive immune responses within the organ—*tissue-associated immunologic ignorance*, *peripheral tolerance to ocular antigens*, and *existence of an intraocular immunosuppressive microenvironment*.

Corneal transplantation is the classic example of ***tissue-associated immunologic ignorance*** and is responsible for success of this procedure. The expression of MHC class I antigens is reduced on corneal epithelial cells and endothelial cells [28], and no corneal cells express MHC class II antigens, so that the lack of MHC class II APCs in the cornea contributes to its success in transplantation [6, 8, 9, 29–35]. Although adult photoreceptor cells and RPE are immunogenic and can induce a

host immune response to retinal autoantigens, the reduced expression of MHC on neurons such as photoreceptors compared to RPE is responsible for their decreased immunogenicity [35, 36].

Peripheral tolerance to ocular antigens contributes to the presence of immune privilege in the eye. Antigen-specific regulatory T cells (T reg cells) and tolerogenic APCs are important components of this mechanism. Two recent reviews by Taylor [26] and Xu [27] provide an in-depth review of the novel mechanisms in the eye responsible for ocular immune privilege. Three APCs in the retina (microglia, perivascular macrophages, and dendritic cells) may contribute to the development of peripheral tolerance to ocular antigens [37]. Although a small population of dendritic cells may be present in the retina, their low numbers suggest they probably do not play a major role in the immunogenicity of retinal tissue [38, 39]. Perivascular macrophages assist in the maintenance of retinal vascular homeostasis and may play a role in presenting antigen to the host, but it is retinal microglia that appear to play the most significant role in retinal pathology [40]. The role of microglia as APCs and effector cells is under active investigation and will undoubtedly contribute to our understanding of their role in regulation of the ocular immune response and whether modulation of their activity can alter the natural course of destructive pathophysiology.

The intraocular inflammatory response is also regulated by an ***immunosuppressive microenvironment*** within the eye that is quite complex and has recently been excellently reviewed by Taylor [26] and Xu [27]. Multiple soluble factors regulate both the innate and adaptive immune response including vasoactive intestinal peptide, somatostatin, and α-melanocyte-stimulating hormone, which regulate the adaptive immune response, and calcitonin gene-related peptide, macrophage migration-inhibitory factor, and soluble CD 95 L, which regulate the innate immune response. Retinal cells also contribute to the immune regulatory system within the retina through expression on their surface of immune modulators such as CD200-CD200R and CX3CL1-CX3CR1, which can suppress immune cell activation, as well as Fas ligand (FasL) and TRAIL (TNF-related apoptosis-inducing ligand), which can induce the death of infiltrating immune cells.

Another important component of the immunosuppressive microenvironment within the eye is the complement system including soluble inhibitors of complement activation, as well as the complement C3 activation product iC3b which is important in the production of TGF-β2 and IL-10 [41, 42]. Multiple complement factors are expressed on the retina and RPE of man [43] and mouse [44]. Both retinal microglia and RPE express complement regulatory proteins CD46, CD55, CD59, and Crry [45–52] that prevent inadvertent activation of the complement system and the destruction of functional tissue. Under physiologic conditions the complement system is constantly activated at a low level (through the alternate pathway), and harmful effects are prevented by various soluble and membrane-bound regulatory molecules [49, 50]. Its low level of activation serves a protective function under normal conditions, but the complement cascade can cause damage to intraocular tissue if not regulated appropriately. It is important to also recognize that expression of complement and complement regulatory genes by cells can be modulated by

cytokines such as TNF-α, IFN-γ, and IL-27 [53, 54], as well as by the supernatants of macrophages [55].

The blood-retina barrier is formed by tight junctions between RPE cells, which protect the outer retina, and retinal vascular endothelial cells, which protect the inner retina. Although this barrier protects the retina from potential destructive pathogens, it does not sequester retinal antigens from recognition by the host immune system as originally thought but contributes to the development of immune privilege in the vitreous cavity and subretinal space [12, 56–58]. The absence of classical lymphatic drainage channels within the neurosensory retina also contributes to the immune privilege of this tissue. Although the choroid does not demonstrate lymphatic drainage in the normal eye, tissue lymphatic channels develop in the choroid with the onset of inflammation or tissue destruction [59, 60].

Although inflammation can disrupt the blood-retina barrier, as well as open dormant lymphatic vessels within the choroid, it is important to realize that the immunosuppressive microenvironment within the eye can still be maintained during and after the resolution of intraocular inflammation. Although alteration of the blood-retina barrier during inflammation allows plasma proteins to enter the eye and degrade immunosuppressive neuropeptides, an immunosuppressive milieu can be reestablished by local activated macrophages that convert latent TGF-β2 to its active immunosuppressive form [61–64]. Immune privilege is a mechanism that evolved to protect the eye from destruction by infectious pathogens. However, it has been of significant clinical benefit for the transplantation of corneal tissue and may have a similar role for retinal transplantation.

Immune Response to Alloantigens [65]

The transplantation of tissue to replace or repair organs has emerged as an important therapeutic option in ophthalmology, as well as in many other fields. Although full thickness corneal transplantation is performed less frequently now for corneal disease, because of the emergence of new surgical options, it still remains the foremost example of successful tissue replacement. Nevertheless, the adaptive immune response to the alloantigens on grafted tissue is still an impediment to corneal transplant success in many patients.

If tissue is grafted between different sites on the same person or between genetically identical subjects (i.e., an autograft), there is complete success. However, when tissue is grafted between unrelated subjects (i.e., an allograft), a T cell-mediated adaptive immune response occurs resulting in acute rejection usually within 2 weeks. The targets of the immune response are cell surface proteins on the grafted tissue (i.e., alloantigens), most frequently associated with MHC class I genes of the donor. These non-self-MHC molecules are recognized by antigen-specific T cells, and thus, MHC loci are the most potent trigger of initial graft rejection. Although MHC matching of recipient and donor was important at the onset of

the organ transplant era, it has become less relevant today for two reasons—first, mHC as well as non-MHC proteins can both incite a rejection reaction; second, there have been major advances in immunomodulatory therapy. When donor and recipient are identical at the MHC but differ at other genetic loci, graft rejection will occur although much more slowly.

Recipient effector T cells that cause graft rejection are activated by APCs. Organ grafts carry APCs of donor origin, e.g., passenger leukocytes, that leave the graft tissue and migrate to secondary lymphoid tissues where they can activate host T cells (i.e., direct allorecognition). Cell transplants avoid this problem since preparation and harvesting of the transplanted cells removes passenger leukocytes. However, an alternative mechanism of alloantigen recognition is the uptake of allogeneic proteins by the recipient's own APCs and presentation to their T cells by self-MHC molecules (i.e., indirect allorecognition). Graft rejection by this mechanism is chronic and frequently involves other cell types (e.g., macrophages) and alloantigenic antibodies.

Hyperacute graft rejection mediated by alloantibodies can occur in vascularized organ grafts since these antibodies most frequently react with antigens on the vascular endothelium of the graft. Cell suspensions or tissues such as photoreceptors and RPE cells that lack vascular endothelial cells are not susceptible to hyperacute rejection. Tissue transplants between species (i.e., xenografts), for example pig to human, are hindered by both human antibodies that react with a ubiquitous cell surface carbohydrate antigen (α-Gal) on pigs, and the less effective function of pig complement regulatory proteins such as CD59, DAF (CD55), and MCP (CD46). The development of transgenic pigs that lack α-Gal and/or express human DAF may minimize these problems in the future and provide a universal source of organ, tissue, and cellular transplants.

Stem Cells and Alloantigens

Pluripotent stem cells are undifferentiated cells capable of indefinite self-renewal and can be differentiated into the three germ layers—endoderm, mesoderm, and ectoderm [66]. The ability to reprogram somatic cells such as fibroblasts into induced pluripotent stem cells (iPSCs) by transfection of four factors—OCT-4, c-Myc, Sox-2, and Klef-4—was first described in 2007 by Takahashi [67]. These reprogrammed cells are pluripotent and are similar in many ways to human embryonic stem cells. Additionally, iPSCs are not associated with the ethical concerns raised by the destruction of a human blastocyst. Unfortunately, HLA expression poses a major hurdle to transplantation of iPSCs [68], the same as for tissues and organs [69, 70]. However, the recent observation that iPSC-derived RPE from MHC-matched donor and recipient can be successfully transplanted into the normal subretinal space of the mouse without provoking an allograft immune response [71] is very important.

Immune suppression has allowed allogeneic transplantation across HLA mismatches, but long-term immunosuppressive regimens are associated with drug toxicity, and antigen nonspecific suppression of the host immune response presents an increased risk for opportunistic infections and malignancy [72–76]. Alternatives to immune suppression include the infusion of regulatory dendritic cells, mesenchymal stem cells, and T reg cells [77]. The generation of HLA universal donors, such as pigs, may be achievable with silencing of HLA expression on transplanted cells or tissue to prevent rejection. Essentially, silencing HLA expression results in a state of immune ignorance in which T cells cannot recognize HLA molecules on grafted cells. It has been reported that human iPSCs may provoke a different immune response depending upon the cell type used for generation. For example, RPE differentiated human iPSCs are tolerated in non-ocular sites better than smooth muscle-derived iPSCs [78].

The isolation of embryonic stem cells (ESC) by Thomson et al. opened the path to exciting potential therapeutic applications in cell replacement therapy [66]. However, one of the major obstacles to their application remains the immune response to allogeneic cells. Thus, various strategies for avoiding allogeneic rejection and inducing immune tolerance have been pursued.

It was initially presumed that ESC may exert some level of "immune protection" from the early observations that these cells expressed low levels of MHC molecules [79], survived transplantation without immune suppression for prolonged periods, and failed to directly stimulate the in vitro proliferation of T cells [80–82]. However, the differentiation of ESC into embryoid bodies in vitro or teratomata in vivo resulted in a significant increase in expression of MHC molecules and evoked a stronger immune response than undifferentiated ESC [83, 84]. Nevertheless, the secretion by ESC of various factors that can suppress the immune response and induce tolerance, e.g., TGF-beta2 and Arginase I, may promote their survival [85–87]. Thus, it appears that ESC and ESC-derived progenitor cells may have the ability to exert immunosuppressive effects under certain conditions, including the site of transplantation, so that the contradictory results in several studies concerning the "immune protection" of ESC may be real.

Both the innate and adaptive immune responses are important in the transplantation of ESC-derived progenitor cells. Natural killer (NK) cells and pattern recognition receptors (PRR), such as Toll-like receptors [TLR], are important mediators of the innate immune response to ESC. TLR ligand expression on ESC is upregulated in response to damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) [88]. However, as in the transplant rejection of allogeneic organs, the adaptive immune response mediated by T cells is of prime importance in ESC survival [89, 90]. ESC-derived progenitors are susceptible to allogeneic rejection across both MHC and mHC mismatches primarily by CD4+ T cells [85, 91], but alloantigen-stimulated CD8+ T cells are also capable of such activity [92]. In addition to histocompatibility antigens the human immune system can also recognize the transcription factor OCT4 that is critical to the pluripotency of ESC cells, suggesting that ESC-derived progenitors may activate the immune response via this molecule [93].

Novel strategies are being developed to prevent immune rejection of human ESC through molecules such as CTLA4-Ig, which disrupts T cell costimulatory pathways, and PD-L1, which activates T cell inhibitory pathways [94]. In conjunction with conventional immune suppression, these strategies may be able to significantly improve the survival and functional integration of human ESC-derived cells into the retina of a recipient since the subretinal space has many characteristics of immune privilege [95]. Long-term survival of postmitotic rod photoreceptor precursor cells after transplantation into the subretinal space of the mouse was observed to be compromised by the early host immune response. However, immune modulation with cyclosporine resulted in the survival of those transplanted cells that escaped damage from the initial immune response [96, 97]. Nevertheless, as described below, the functional effect of transplanted photoreceptor cells in mice is probably not related to their anatomic integration into the neurosensory retina and the establishment of functional bipolar cells synapses.

Regenerative Medicine

Enormous interest in the treatment of inherited retinal diseases (IRD) using tissue or stem cell transplants to replace or regenerate RPE and/or photoreceptor cells exists today. However, recent observations in two laboratories question the feasibility of retinal transplantation to replace photoreceptor cells since the effects observed so far in mice seem to be the result of transfer of cytoplasmic information from donor cells to host cells, and not due to integration of transplanted cells into the neurosensory retinal network [98, 99]. Material transfer does not involve permanent donor-host nuclear or cell-cell fusion, with the uptake of free proteins or nucleic acid from the extracellular environment. Instead, RNA and/or protein are exchanged between donor and host cells in vivo. Although this is a formidable obstacle, there are other challenges as well, including the very few transplanted photoreceptor cells that integrate into the retina [100, 101], as well as immunologic rejection of grafted tissue. The question of immune privilege in the subretinal space and transplanted tissues/cells still needs clarification although there is evidence it exists in the normal subretinal space [12, 13, 102, 103]. Investigative studies need to be performed exploring immune privilege in models of IRD with a comparison to the normal subretinal space. Additionally, various types of transplanted tissues need to be studied including fetal retinal tissue, retinal-differentiated induced pluripotent stem cells (iPSC), and adult retinal tissue. It is only through a systematic study of both the transplant site and the tissue to be transplanted that the parameters of immune privilege regarding retinal transplantation will be unraveled. A recent important study by Sugita et al. (2016) demonstrated that MHC-matched iPSC-derived RPE cells will survive in the normal subretinal space without being destroyed by the host immune response in contrast to MHC-mismatched allografts.

In both the anterior chamber and the subretinal space immune privilege is a dynamic phenomenon [104]. Transplantation of both retinal fragments and RPE in

mice demonstrated the immunogenicity of both tissues, as well as the existence of immune privilege in both the anterior chamber and subretinal space [103, 105, 106]. However, other experiments suggested that immune privilege in the anterior chamber and subretinal space differs, and that retinal tissue transplants may make a significant contribution to their immune protection from host destruction in the subretinal space [6, 8, 9]. Thus, the survival of transplanted retinal tissue depends upon both the immune privilege status of the site, and the immunogenicity and immune protective mechanisms of the transplant.

The RPE is a tissue that serves many important functions biologically and immunologically. It has the ability to inhibit T cell activation by secretion of TGF-β2, as well as the expression of CD95L, a ligand that can induce T cell apoptosis [107–110]. The innate immunogenicity of neonatal RPE may depend on whether RPE is transplanted as a tissue, where it appears to have immune privilege, or as cell suspensions, where it does not. In studies of the immune protection provided by transplanted neuronal retina, it was observed that both syngeneic and allogeneic *adult* retinal tissue did not survive transplantation. In contrast, *neonatal* retinal tissue survived transplantation, but allogeneic retinal tissue was ultimately disorganized because it elicited a host immune response [103, 105, 106]. As stated previously there is evidence that tissue-associated immune privilege exists in some differentiated iPSC [111].

The normal subretinal space exhibits immune privilege, but it is a dynamic phenomenon that can be abrogated by inflammation or disruption of the blood-retinal barrier. The immune privilege of the subretinal space in IRD needs further investigation to define the respective roles of innate and adaptive immunity [112]. Additionally, tissue-associated immune privilege of the retinal transplant is also dynamic and can be enhanced or inhibited by cytokines elaborated by parenchymal cells and/or inflammatory cells.

Immunology of Retinal Transplantation in Humans

RPE and photoreceptors are the two major cell types considered for transplantation in IRD. Two recent excellent reviews of cell-based therapy for degenerative retinal diseases by Zarbin et al. discuss retinal transplantation in depth [113, 114]. Consequently, the following discussion will focus on the immunology of retinal transplantation performed in man. Many different techniques have been used to place RPE beneath the fovea in patients with exudative age-related macular degeneration (wet AMD). Most frequently, patients with wet AMD underwent submacular scar excision with transplantation of either fetal or adult sheets of RPE [115–117]. We observed that immune suppression was required to prevent rejection of allogeneic RPE sheets harvested from cadaveric donor eyes [117]. Our study confirmed that allogeneic RPE is immunogenic and can disrupt the immune privilege of the subretinal space in AMD, an observation reported by others [103, 116–121].

Several trials have transplanted RPE monolayers or cell suspensions in nonexudative AMD (dry AMD) with mostly poor results [122–125]. It is important to recall that geographic atrophy (GA) involves loss of both RPE and photoreceptors, and thus, RPE transplants in large areas of GA should not be expected to restore central vision unless viable photoreceptors are present nearby. Most recently, the targeting of border areas between viable and nonviable photoreceptors on the edge of GA for RPE transplantations has been studied. Schwartz et al. [126, 127] reported the 22-month and 4-year results of human embryonic stem cell (hESC)-derived RPE cell suspensions for GA in AMD and in Stargardt disease, an IRD caused by an autosomal mutation in ABCA4 or ELOV. Early results were encouraging and relatively safe—no treated eyes lost vision, all transplants were well tolerated [126], and the results were sustained up to 4 years [127]. Unfortunately, this study does not clarify the existence of immune privilege in the subretinal space in these diseases. All patients were started on an immunosuppressive protocol for 12 weeks—tacrolimus and mycophenolate for six weeks, followed by mycophenolate alone for an additional six weeks. Additionally, it is unknown if the transplanted hESC survived. Although no retinal inflammation was observed, it is possible that a host immune response without observable cellular inflammation destroyed the transplanted cells. The presence of melanin pigment in the area of transplant could either be in transplanted cells or in macrophages that have ingested destroyed transplanted cells.

The rationale for photoreceptor transplantation is twofold. First, it is well known that the presence of rod photoreceptor cells promotes cone photoreceptor survival. Thus, rod-rich transplants might theoretically rescue cones or at least allow still functioning cones to survive. Second, photoreceptor transplants might synapse with functioning bipolar cells in the inner retina, thereby re-establishing the normal retinal circuitry. The anatomy of the inner retina in retinitis pigmentosa (RP) is maintained until very late in the disease (albeit with significant synaptic sprouting exhibited by residual viable neurons), and even when disorganized it maintains the ability to send visual percepts to the occipital cortex [128–132].

Sheets of both fetal and cadaveric human photoreceptors have been transplanted [133–136], but no subretinal transplants of photoreceptor cell suspensions have been reported in man. While both fetal and cadaveric tissue were safe and well tolerated, none of these studies showed improved visual acuity or electrophysiology. Several pieces of evidence suggest that photoreceptor transplants in the subretinal space can remain viable for prolonged periods of time. Kaplan et al. [133, 137] did not observe evidence of a host allograft reaction (i.e., chorioretinitis, vitritis, or CME) to photoreceptor cells even though patients were not immune suppressed, in contrast to RPE transplants [117]. This observation is consistent with the decreased immunogenicity of photoreceptor cells [138–142], as well as the possible existence of immune privilege in the subretinal space even in advanced RP. We have observed the survival of transplanted human adult xenogeneic photoreceptor sheets within the subretinal space of the normal nonhuman primate 6 months after the use of systemic immune suppression for a short period postoperatively (unpublished observations).

In a single series of combined fetal RPE and photoreceptor transplantation, seven of 10 patients received no systemic immune suppression and were observed to have no evidence of immune rejection [136]. A modest improvement in visual acuity was observed; however, these results have not been duplicated, and the viability of the transplant is in question since pigmented RPE in the transplant disappeared during the course of follow-up [143].

The major barriers for photoreceptor stem cell therapy in advanced IRD are the large numbers of transplanted cells that would be required to integrate into the retina, as well as their ability to create functional synapses with residual bipolar cells to reestablish the inner retinal neural network. To date there is only limited success in vivo with regard to the efficient integration of transplanted photoreceptor cells into the retina. In most studies in mice, only 0.5% of transplanted photoreceptor cells integrated with host retina [101]. Furthermore, as mentioned above, it is no longer clear that the effects of photoreceptor cell transplantation in mice are the result of cellular integration and synapse formation, rather than cytoplasmic exchange between donor and host cells [98, 99]. Although graft rejection by the host is a significant consideration, immunosuppressive protocols that are now effective in organ transplantation could certainly be employed if the grafted tissue showed significant evidence of functional improvement for the host.

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Chapter 9
Restoration of Cone Photoreceptor Function in Retinitis Pigmentosa (RP): Retinal Cell-Based Therapy

Henry J. Kaplan, Wei Wang, and Douglas C. Dean

Cone Dormancy in RP

Retinitis pigmentosa (RP) is the major cause of hereditary blindness in North America. It is a group of inherited retinal diseases clinically characterized by the onset of night blindness, early loss of peripheral vision, and late loss of central vision. Most patients with this disease are able to maintain functional vision for many years, until the late stages of the disease when retinal degeneration approaches the macula and cone degeneration ensues, resulting in the loss of central vision. If therapeutic intervention could prevent or reverse the onset of cone degeneration within the macula patients would be immeasurably helped.

Over 67 mutant genes (www.sph.uth.tmc.edu/Re tNet/home.htm) have been identified in monogenic forms of RP, all leading to a similar clinical phenotype, with over 95% of those mutations in rod-specific genes. Many of these mutations arise in genes such as rhodopsin (RHO) that play a direct role in Rod visual transduction. Mutant RHO becomes trapped in the endoplasmic reticulum and rods undergo apoptosis [1–3]. With rod degeneration, cone photoreceptors begin to lose function highlighted by the loss of functional structures, including visual pigment-rich outer segments (OS) and mitochondrial-rich inner segments (IS). In contrast to rod cell bodies that die during rod photoreceptor degeneration there is long-term persistence of cone cell bodies after degeneration of cone OS and IS, resulting in persistent cone nuclei in RP patients. The presence of viable cone nuclei in RP is termed cone dormancy [4–8] (Fig. 9.1).

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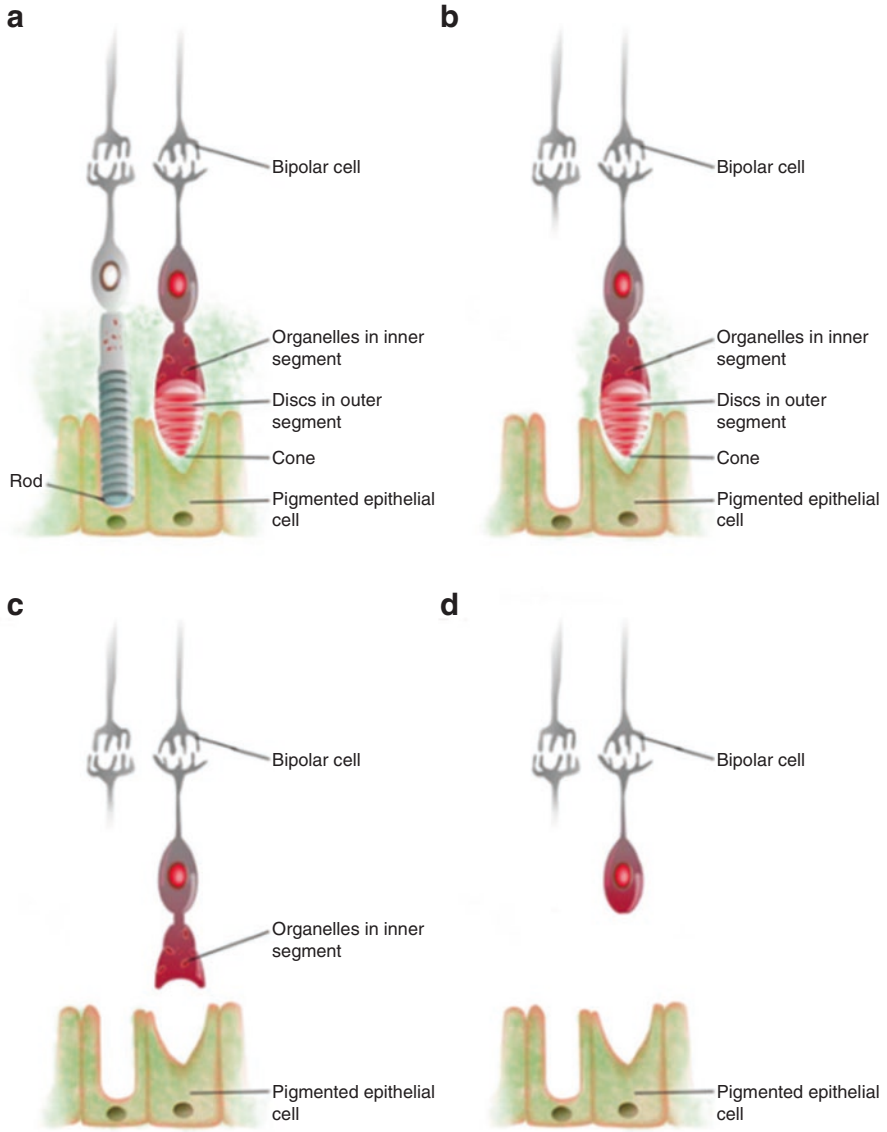

Fig. 9.1 Cone dormancy in RP results in viable cone nuclei for many years after OS/IS degeneration. (**a**) Glucose (*green dots)* transport to the subretinal extracellular space and cone IS is essential to provide energy for regeneration of cone OS. (**b**) Degeneration of ROS and loss of contact with apical microvilli results in entrapment of glucose in RPE. Ensuing glucose starvation of cone photoreceptors results in OS degeneration (**c**) and IS disassembly but with maintenance of viable cone nuclei (**d**). *ROS* rod outer segment

Reactivation of Dormant Cones in RP by Rod Precursor Transplants

Photoreceptors are among the most metabolically active cells, and like other neurons depend on glucose for energy production, as well as OS synthesis [9, 10]. Inhibition of the essential glycolytic pathway enzyme glyceraldehyde 3-phosphate dehydrogenase with iodoacetic acid results in rapid loss of OS in both rods and cones [11]. As the glycolytic block diminished, dormant cones resumed OS synthesis and function but rods failed to do so and died. We developed a pig model of the most common autosomal dominant RP in North America, a Pro23His mutation in Rhodopsin, referred to as P23H retinopathy [12, 13]. Cones are concentrated in a visual streak in the pig and transition to dormancy with rod degeneration [14].

Since cone degeneration in RP follows the demise of mutant Rod photoreceptors we asked if transplantation of wild-type (WT) rod precursors might preserve endogenous cone 0S and electrophysiology in P23H pigs [15]. 5×10^5 rod precursor cells were injected into the subretinal space beneath the visual streak at P 14 and the recipients assessed at P60. We found the transplanted cells formed monolayers of RHO^+ OS extending to the RPE and maintained cone OS out to a distance of 1000 μm from the transplant site. Cone electrophysiology using the photopic multifocal ERG (mfERG) was increased in regions surrounding these transplant sites (Fig. 9.2). Thus, transplantation of WT Rod precursors into the visual streak at P 14 delayed transition of cones to dormancy in regions surrounding transplant sites in our pig model of P23H retinopathy. This effect was linked to the ability of the transplanted cells to form a monolayer and extend RHO^+ OS toward the RPE. When WT rod precursor transplants were performed in the late stage of cone dormancy, when OS are degenerated and IS are disassembled, we observed restoration of IS assembly and reactivation of OS synthesis, with return of the mfERG [15].

Cone dormancy in RP Results from Glucose Starvation

Since glucose is such an important metabolic fuel for photoreceptors, we wondered if these transplanted rod precursors might be affecting access of dormant cones to glucose. We initially examined expression of Txnip, the most highly glucose-inducible gene identified to date, which regulates the balance between anaerobic versus aerobic metabolism [16, 17, 18]. We observed that Txnip was induced at birth in WT pigs in the ellipsoid of cone IS, where mitochondria are concentrated, and persisted in adulthood. Txnip was not detected in rods or RPE. In contrast to WT pigs, Rod OS are lost following birth in the P23H retina and Txnip was not identifiable in cones of P23H pigs. Interestingly, Txnip was induced in endogenous cone IS following Rod precursor transplants. These observations provided initial evidence that cone access to glucose in vivo is dependent on WT rods.

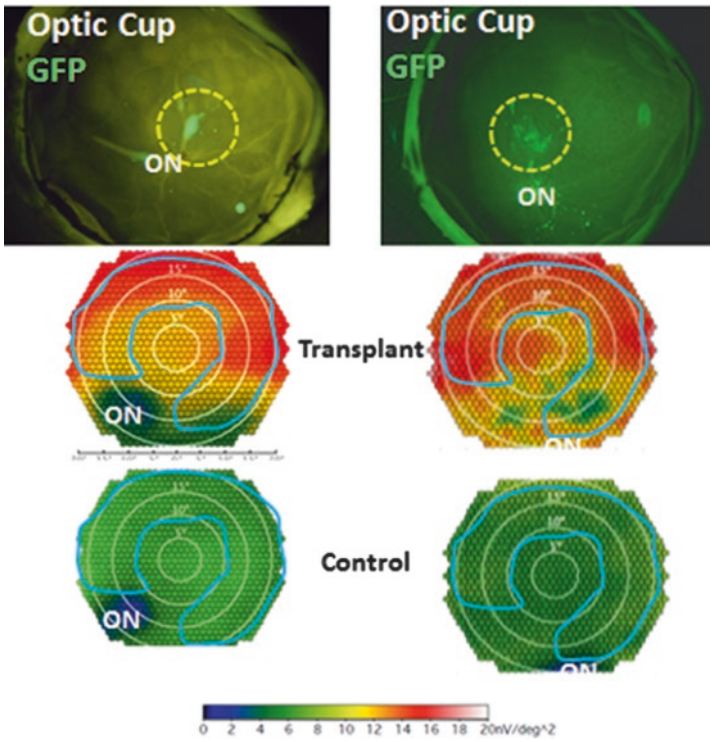

Fig. 9.2 Transplantation of GFP+ rod precursors into the subretinal space of P23H pigs preserves photopic mfERG. Above, positions of injected GFP+ rod precursors just superior to the optic nerve (ON) and the region of mfERG assessment (yellow circle) in the optic cup is shown. Below, representative mfERGs showing increased local photopic mfERGs linked to preservation of COS in the region surrounding transplants are shown

Glucose Is Sequestered in the RPE

We followed glucose transport from the circulation into photoreceptors in vivo using fluorescently labeled 2-deoxy-glucose. Glucose is transported to the outer retina from the choroid to the RPE, and then from the RPE into the subretinal space where it enters photoreceptors via Glut1 receptor complexes on IS [19]. Mice with the P23H Rho mutation were injected in the tail vein with fluorescently labeled glucose at the onset of Rod degeneration (P34). After 1 h, labeling of the inner retina appeared similar in the WT and mutant mice; however, there was a dramatic difference in labeling of the outer retina (Fig. 9.3). Both rod and cone IS were highly labeled in the WT retina, but were not labeled in the P23H retina. By contrast, the RPE was highly labeled in the P23H retina, whereas little labeling of RPE was evident in the WT retina. Thus, with onset of Rod OS shortening, glucose accumulates in the RPE and is no longer transported to cone receptors in the P23H retina.

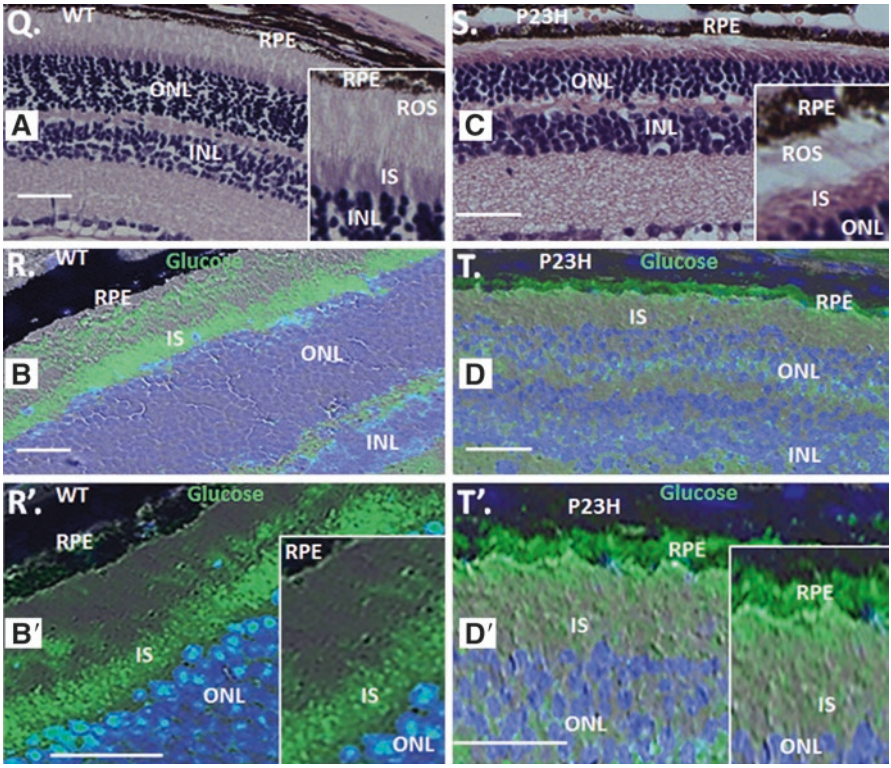

Fig. 9.3 (**A–D′**) Fluorescent 2-deoxy-glucose was injected in the tail vein of P23H Rho knock-in mice (**C–D′**) ($n = 4$) and WT littermates (**A–B′**) ($n = 4$) at P35, and frozen eye sections were analyzed 1 h later. Bars are 30 μm

Subretinal Glucose Replacement Reactivates Cone OS Synthesis in RP

Since glucose becomes sequestered in the RPE and is not delivered to photoreceptors in the P23H retina, we investigated if injection of glucose into the subretinal space beneath the visual streak would bypass its entrapment in the RPE and reverse cone dormancy. We injected glucose at a concentration of 280 mM in a 50 μL volume in P23H pigs when rods are lost and cones retain IS (P45). After 3 days we found that Opsin$^+$ cone OS were restored for approximately 1500 μm from the injection site, with the induction of Txnip in the area of cone OS restoration. Our results suggested that glucose in cones is being utilized for OS synthesis. Since long-chain fatty acids are essential components of OS synthesis we investigated and documented the induction of several key enzymes, such as fatty acid synthase and acetyl CoA carboxylase, as well as the miRNA 96/182/183 cluster transcript (Fig. 9.4).

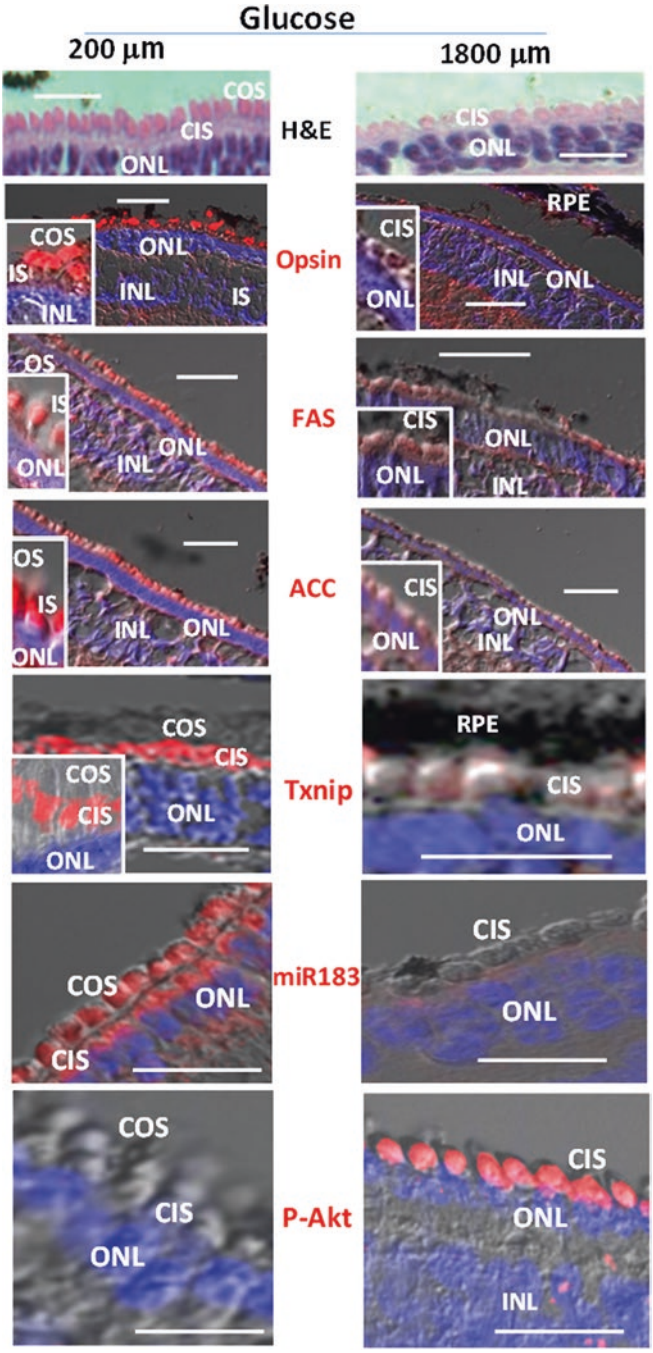

Fig. 9.4 Glucose was injected at P45, and eyes were examined 3 days after injection. Sham indicates injection of control media into the contralateral eye. Bars are 40 µm. Representative immunostaining after glucose injection showing that OS are restored and Txnip, FAS, ACC, and miR-183 are induced in endogenous cones in the region of the injection site

These studies suggested retention of glucose by RPE in the P23H retina causes loss of cone OS synthesis, leading to dormancy. Glucose provides metabolites for cone OS formation, as well as induction of fatty acid synthesis pathways which directs these metabolites toward OS synthesis.

Regulation of Glucose Metabolism in Cones

Pyruvate dehydrogenase (PDH) is the "gatekeeper" for aerobic glycolysis and catalyzes acetyl CoA production from glucose-derived pyruvate for fatty acid synthesis. However, acetyl CoA is also the entry point into the TCA cycle, where resulting isocitrate and malate act as precursors for most of the NADPH used in the visual cycle [20]. The PDHε subunit is inhibited by Akt-dependent phosphorylation to block mitochondrial metabolism and drive glucose-derived pyruvate into anaerobic metabolism for ATP production in cancer [21–23]. We found phosphorylated Akt (P-Akt) accumulated in P23H cones, where it was concentrated in IS and that following glucose injection into the subretinal space P-Akt decreased in cones in parallel with glucose induction of Txnip and restoration of OS synthesis. Our studies with AAV Txnip shRNA confirmed that induction of Txnip in cones prevents constitutive Akt activation, thereby allowing glucose metabolites to be diverted to aerobic metabolism, and then induction of the fatty acid synthesis pathway directs these metabolites toward generation of cone OS [15].

Conclusions

Most mutations in RPE occur in Rod-specific genes, so that the accompanying loss of cone function has been puzzling. Primary cultures of cones show that purified RdCVF added to the cultures can bind a Glut1 complex on cone IS and promote glucose uptake into the cells [19]. Because RdCVF is expressed by rods, loss of its expression following rod degeneration might therefore restrict glucose uptake into cones. We observed diminished mutant rod OS extension to retention of glucose in the RPE, thereby preventing its transport to both persistent mutant rods, as well as cones. Indeed, directly blocking rod OS formation by mutating the OS protein rds/peripherin [24] leads to RP with cone dormancy, supporting such a role for rod OS loss in initiation of RP. Overexpression of RdCVF in the subretinal space would be expected to drive uptake of diminishing levels of glucose in the subretinal space into cones as glucose becomes sequestered in the RPE, thereby delaying transition to cone dormancy. Similarly, enhancing photoreceptor glycolysis by mutation of Sirt6, which also promotes glucose uptake into cones, will also only delay transition to cone dormancy. Once glucose is sequestered in the RPE and cone OS synthesis is lost, neither RdCVF nor Sirt6 is capable of reinitiating OS synthesis and reversing

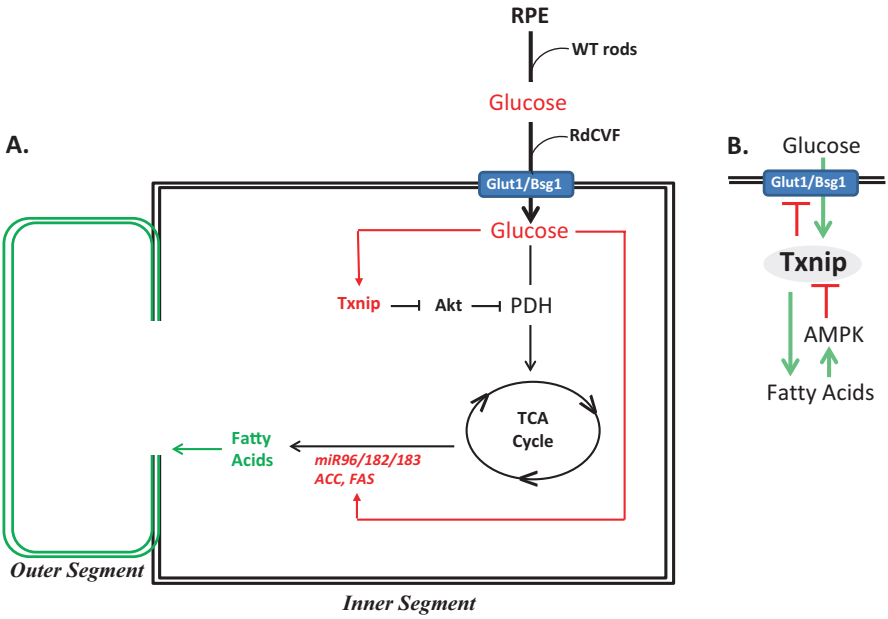

Fig. 9.5 Transplant of rod precursors restores access of cones to glucose in the P23H retina where glucose in turn regulates its own metabolic pathway. (**A**) Proposed pathways in rod-mediated reactivation of end-stage dormant cones in RP. (**B**) Txnip as a regulator of glucose metabolism to fatty acids

dormancy. Reversal of dormancy requires either rod transplantation to reestablish glucose transport or glucose injection to bypass its retention in the RPE.

Glucose starvation of cones in the visual streak during RP leads to loss of functional structures. Yet, the cells survive in a dormant state with little more than a nucleus persisting. We observed that cone dormancy is highlighted by constitutive Akt activation, to inhibit PDH activity, and thereby drive anaerobic metabolism which is responsible for loss of cone OS synthesis. The PDHε subunit is inhibited by Akt-dependent phosphorylation to block mitochondrial metabolism and drive glucose-derived pyruvate into anaerobic metabolism in cancer [21–23]. Txnip is required to prevent this inhibitory phosphorylation, thereby promoting aerobic conversion of glucose-derived pyruvate into acetyl CoA for production of fatty acids and NADPH [16, 18] (Fig. 9.5). Although Txnip is induced by glucose, it feeds back to inhibit activity of Glut1, thereby limiting glucose uptake [16–18]. In this way Txnip tightly regulates the level of glucose in the cell.

Approaches aimed solely at neuroprotection or regulating metabolic pathways within cones will ultimately fail to preserve cone photoreceptors in RP because glucose transport from the RPE to photoreceptors is diminished in RP. We have observed two approaches to addressing this problem with glucose starvation—the subretinal transplant of WT rods to restore glucose transport from the RPE, eliminating the

problem in the region of cell transplant; replacement of glucose in the subretinal space. Although neither approach has immediate clinical application, a better understanding of the intracellular pathways responsible for the transport of glucose from the RPE to the subretinal space may well provide a pharmacologic approach to reversal of glucose starvation.

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Chapter 10
Cell Delivery: Surgical Approaches

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Introduction

Retinal Degeneration and the Need of New Treatments

Retinal degenerative diseases as a group constitute one of the primary causes of permanent visual impairment, affecting millions of people worldwide. The effect of this group of conditions is debilitating with a major impact on a patient's daily life including difficulty in performing basic functions, deterioration of personal independence, and often an effect on mental health. Among the most prevalent retinal degenerative diseases

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are age-related macular degeneration (AMD) and inherited retinal dystrophies of which retinitis pigmentosa (RP) and Stargardt disease (STGD) are the commonest.

AMD represents the third leading cause of legal blindness and the most prevalent cause of permanent visual loss in the over 55 years age group worldwide [1]**.** RP constitutes the leading cause of inherited blindness estimated to affect approximately 1/4000 individuals [2], and STGD is the most common juvenile retinal degenerative disease, with a prevalence of 1/8000–10,000 young individuals. [3]

The eye has been identified as one of the most amenable organs to be targeted by the first generation of regenerative medicine techniques. It is easily accessible surgically, and there are multiple imaging modalities using only light sources which provide the ability to document structural and functional outcomes with minimal risk. Additionally, the eye and especially the vitreous and subretinal space is a relatively immune-privileged site, theoretically able to tolerate foreign antigens or non-histocompatible cells without eliciting an immune response. Hence, under normal circumstances the risk of tissue rejection after cell transplantation is reduced. Furthermore, it is a small organ, and the majority of retinal degenerative diseases initially target one type of cell (retinal pigment epithelial (RPE) cells, photoreceptors, ganglion cells, etc.), in a way that cell therapies can be focused on replacing one specific cell group by transplanting a relatively small number of cells. These advantages together with the invaluable combination of established surgical experience and current development in experimental retinal surgery have put retinal degenerative diseases at the forefront of cell-based clinical research.

In addition to the imaging and access advantages of the eye, progress in laboratory methods of differentiation and cultivation has increased the availability of various types of potentially therapeutic cells (Table 10.1). As a result, numerous clinical trials involving retinal and RPE transplantation have commenced worldwide, some of which show encouraging preliminary results, in terms of safety and possible efficacy (Tables 10.2, 10.3, and 10.4).

Therapeutic Formulations of Cell Therapies

Cell Suspension

A cell suspension consists of a liquid medium—usually balanced salt solution or other optimized aqueous medium—in which single cells or small aggregates of cells are floating. Ideally, the cells would have undergone differentiation, isolation, purification, and characterization, so that only the desired cell type is included in the suspension.

A cell delivery method in the form of suspension holds the major advantage that it requires a relatively minor surgical intervention. Cells can easily be injected in the intravitreal or subretinal space via small gauge cannulas, causing only minimal or no injury to the retina.

Currently the most common approach for implanting a cell suspension is subretinal delivery via the pars plana, i.e., the transvitreal route (Fig. 10.1c). This approach requires a standard pars plana vitrectomy and transretinal access to the

Table 10.1 Therapeutic cells: definitions and classification

Category	Definition
Stem cells (SC)	Cells in undifferentiated state, capable of infinite proliferation and able to differentiate into various cell types
Totipotent SC (a.k.a. omnipotent)	Cells capable of differentiation into both embryonic and extraembryonic cell types. Able to generate a complete, viable organism
Pluripotent SC	Cells capable of differentiation and tissue generation of any of the three embryonic germ layers, i.e., ectoderm, mesoderm, and endoderm
Multipotent SC	Cells capable of differentiation into limited cell types, able to generate tissue of a single germ layer
Oligopotent SC	Cells capable of differentiation into only a few cell types, e.g., myeloid, lymphoid SC
Unipotent SC	Cells capable of differentiation only into their own cell type, but retain ability to self-renew
Human embryonic SC (hESC)	Pluripotent SC obtained from a 5-day-old blastocyst
Induced pluripotent SC (iPSC)	Pluripotent SC obtained by adult somatic cells by dedifferentiation through genetic reprogramming
Mesenchymal SC (MSC)	Multipotent stromal cells capable of differentiation into variable cell types, i.e., chondrocytes, myocytes, adipocytes, and osteoblasts
Adipose derived SC (ASC)	Series of MSC derived from adipose tissue, capable of differentiation into endodermal, mesodermal, and ectodermal tissues
Human umbilical tissue-derived cells (hUTC)	Series of MSC derived from human umbilical cord tissue
Hunan retinal progenitor cells (hRPC)	Partially differentiated cells obtained from fetal neural retina, capable of differentiation into retinal cell, but not for infinite replication

subretinal space. Less common, but also less invasive is the intravitreal injection, which does not necessitate surgery in the operating room, but only a simple transscleral injection of the suspension into the vitreous cavity (Fig. 10.1b). Finally, a completely different method uses an "external" approach and a purpose-designed micro-catheter to deliver the cell suspension through the sclera and choroid into the subretinal space (Fig. 10.1a). A more detailed description of these methods will be given in the next section of this chapter.

Sheets/Patches

A cell sheet/patch transplant system consists of a biocompatible substrate or scaffold, seeded with the therapeutic cells in a way that they form a cellular monolayer (e.g., a RPE monolayer patch). The scaffold provides the supportive surface necessary for the cells to attach, proliferate, differentiate, and meet their structural and functional roles after transplantation. Additionally, the artificial membrane provides the required structural rigidity for the manipulations during the delivery process.

Table 10.2 Recent and current cell transplantation studies (Cell suspension approach)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT02467634	Study of HUCNS-SC Subretinal Transplantation in Subjects With GA of AMD (RADIANT)	II	StemCells, Inc.	Various retina and vitreous associates, Arizona, California, Illinois, Michigan, New York, Texas, Utah, US	Dry AMD	HuCNS-SC	Cell suspension—subretinal injection	Jun-2015
NCT02464436	Safety and Tolerability of hRPC in Retinitis Pigmentosa	I/II	ReNeuron Limited	Massachusetts Eye and Ear Infirmary, Massachusetts, US	RP	hRPC	Cell suspension—subretinal injection	May-2015
ChiCTR-ONB-15007477	Clinical study of subretinal transplantation of human bone marrow mesenchymal stromal cells with or without embryonic retinal progenitor cells in treatment of retinal pigmentosa	New Treatment Measure Clinical Study	Southwest Hospital, Shapingba District, Chongqing, China. National Basic Research Program (973 Program)	Southwest Hospital, Third Military Medical University, Shapingba District, Chongqing, China	RP	hBMMSC (Human bone marrow mesenchymal SC) with or without RPC	Cell suspension—subretinal injection	Oct-2015

ChiCTR-OCB-15007054	Clinical study of subretinal transplantation of clinical human embryonic stem cells derived retinal pigment epitheliums in treatment of dry age-related macular degeneration diseases	New Treatment Measure Clinical Study	Institute of Zoology, Chinese Academy of Sciences	Beijing Tongren Hospital, Capital Medical University, China	Dry AMD	hESC-RPE	Not Provided	Sep-2015
ChiCTR-OPC-15006757	Treatment of Dry Age-related Macular Degeneration using fetal retinal pigment epithelium	I	The First Affiliated Hospital of Nanjing Medical University	The First Affiliated Hospital of Nanjing Medical University, China	Dry AMD	Fetal RPE	Not Provided	Jul-2015
ChiCTR-OCB-15006423	Clinical study of subretinal transplantation of human embryo stem cell derived retinal pigment epitheliums in treatment of macular degeneration diseases	New Treatment Measure Clinical Study	Southwest Hospital, Shapingba District, Chongqing, China	Southwest Hospital, Shapingba District, Chongqing, China	Macular Degeneration Diseases	hESC-RPE	Not Provided	May-2015

(continued)

Table 10.2 (continued)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT02320812	Safety of a Single, Intravitreal Injection of Human Retinal Progenitor Cells (jCell) in Retinitis Pigmentosa	I/II	jCyte, Inc.	The Gavin Herbert Eye Institute, Univ California Irvine Retina-Vitreous Associates Medical Group, LA, California, US	RP	hRPC	Cell suspension—intravitreal injection	Dec-2014
NCT02286089	Safety and Efficacy Study of OpRegen for Treatment of Advanced Dry-Form Age-Related Macular Degeneration	I/II	Cell Cure Neurosciences Ltd.	Hadassah Ein Kerem University Hospital, Israel	Dry AMD	hESC-RPE	Cell suspension—subretinal injection	Nov-2014

NCT02280135	Clinical Trial of Intravitreal Injection of Autologous Bone Marrow Stem Cells in Patients With Retinitis Pigmentosa	I	Red de Terapia Cellular, Spanish NHS, Hospital Universitario Virgen de la Arrixaca, Fundacion para la Formacion e Investigacion Sanitarias de la Region de Murcia Public Health Service, Murcia, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca	Clinical Universitary Hospital Virgen de la Arrixaca, Murcia, Spain	RP	Autologous BMSC	Cell suspension—intravitreal injection	Oct-2014
NCT02122159	Research With Retinal Cells Derived From Stem Cells for Myopic Macular Degeneration	I/II	Ocata Therapeutics, University of California	University of California, LA, US	Myopic Macular Degeneration	hESC-RPE	Cell suspension—subretinal injection	Apr-2014

(continued)

Table 10.2 (continued)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT01920867	Bone Marrow Derived Stem Cell Ophthalmology Treatment Study (SCOTS)	Not Provided	Retina Associates of South Florida/ MD Stem Cells	Retina Associates of South Florida, Florida, US; Al Zahra Private Hospital, United Arab Emirates	Retinal Disease, Macular degeneration, Hereditary retinal dystrophy, Optic nerve disease, glaucoma	Autologous BMSC	Cell suspension—retrobulbar, subtenon, intravitreal, intraocular, subretinal and intravenous injection	Aug-2013
NCT01914913	Clinical Study to Evaluate Safety and Efficacy of BMMNC in Retinitis Pigmentosa	I/II	Chaitanya Hospital, Pune	Chaitanya Hospital, Pune, India	RP	Autologous BMMNSC (mononuclear cells)	Cell suspension—Intravitreal injection	Jul-2013
NCT02016508	Intravitreal Injection of Human Bone Marrow Derived Mesenchymal Stem Cell in Patients With Dry Age-related Macular Degeneration (AMD)	I/II	Al-Azhar University	Al-Azhar University, Egypt	Dry AMD	BMSC	Cell suspension—intravitreal injection	May-2013

NCT01674829	A Phase I/IIa, Open-Label, Single-Center, Prospective Study to Determine the Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (MA09-hRPE) Cells in Patients With Advanced Dry Age-related Macular Degeneration (AMD)	I/IIa	CHABiotech CO., Ltd.	CHA Bundang Medical Center, Korea	Dry AMD	hESC-RPE	Cell-suspension—subretinal injection	Aug-2012
NCT01736059	Clinical Trial of Autologous Intravitreal Bone-marrow CD34+ Stem Cells for Retinopathy	I	University of California, Davis	University of California, Davis, California, US	Dry AMD, DR, RVO, RP, Hereditary Macular Degeneration	BMSC	Cell suspension—intravitreal injection	Jul-2012

(continued)

Table 10.2 (continued)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT01632527	Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Age-Related Macular Degeneration (AMD)	I/II	StemCells, Inc.	Retina-Vitreous Associates Medical Group and Byers Eye Institute at Stanford, California; New York Eye and Ear Infirmary, New York; Retina research Institute of Texas and Retina Foundation of the Southwest, Texas, US	Dry AMD	HuCNS-SC (Human neural stem cells)	Cell suspension—subretinal injection	Jun-2012
NCT01625559	Safety and Tolerability of MA09-hRPE Cells in Patients With Stargardt Macular Dystrophy	I	CHABiotech CO., Ltd.	Republic of Korea	Stargardt Macular Dystrophy	hESC-RPE	Cell suspension—subretinal injection	Jun-2012

NCT01531348	Feasibility and Safety of Adult Human Bone Marrow-derived Mesenchymal Stem Cells by Intravitreal Injection in Patients With Retinitis Pigmentosa	I	Mahidol University, Ministry of Health, Thailand	Siriraj Hospital Mahidol University, Bangkok, Thailand	RP	BMSC	Cell suspension—intravitreal injection	Feb-2012
NCT01344993, NCT02463744 (Dry AMD) and NCT01345006 NCT02445612 NCT01469832 (Stargardt)	Safety and tolerability of sub-retinal transplantation of hESC derived RPE (MA09-hRPE) cells in patients with advanced dry age related macular degeneration (Dry AMD) and Stargardt Macular Dystrophy	I/II	Ocata Therapeutics Inc. (Formerly: Advanced Cell Technology Inc. ACT), Astellas Institute for Regenerative Medicine	Jules Stein Eye Institute, California; Bascom Palmer Eye Institute, Florida; Mass Eye and Ear, Massachusetts; Wills Eye Institute, Pennsylvania, US	Dry AMD, Stargardt Macular Dystrophy	hESC-RPE	Cell suspension—subretinal injection	Apr-2011

(continued)

Table 10.2 (continued)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT01226628	A safety study of CNTO 2476 in patients with age-related macular degeneration	I	Janssen Research & Development, LLC	Arcadia, California, Philadelphia, Pennsylvania, US	Dry AMD	hUTC	Cell suspension—subretinal injection	Oct-2010
CTRI/2010/091/00639	A Clinical Trial to Evaluate the Effect of Bone Marrow Derived Stem Cell in Diseases Like Dry Age Related Macular Degeneration and Retinitis Pigmentosa	I	Indian Council of Medical Research, India	All India Institute of Medical Sciences, Ansari Naga, India	Dry AMD, RP	BMSC (mononuclear cells)	Cell suspension—intravitreal injection	May-2010
NCT01560715 NCT01068561 NCT01518127 and NCT01518842	Intravitreal Bone Marrow-Derived Stem Cells in Patients With RP Macular Degeneration and Ischemic Retinopathy	I	University of Sao Paolo	Rubens Siqueira Research Center, Brazil	RP, Dry AMD, Ischemic Retinopathy	Autologous BMSC	Cell suspension—intravitreal injection	Feb-2010 Sep-2011 Jan-2012 Mar-2012

Table 10.3 Recent and current cell transplantation studies (Cell sheet approach)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT02590692	Study of Subretinal Implantation of Human Embryonic Stem Cell-Derived RPE Cells in Advanced Dry AMD	I/IIa	Regenerative Patch Technologies (RPT)	University of California, US	Dry AMD	hESC-RPE	Cell sheet—subretinal implantation	Oct-2015
JPRN-UMIN000011929	A Study of transplantation of autologous induced pluripotent stem cell (iPSC) derived retinal pigment epithelium cell sheet in subjects with exudative age related macular degeneration	Not Provided (Safety/ Exploratory)	RIKEN Institute	RIKEN, IBRI-Kobe Hospital, Japan	Wet AMD	iPSC-RPE	Cell sheet—subretinal implantation	Oct-2013
NCT01691261	A Study of Implantation of Human Embryonic Stem Cell Derived Retinal Pigment Epithelium In Subjects With Acute Wet Age Related Macular Degeneration And Recent Rapid Vision Decline	I	Pfizer, London Project to Cure Blindness, University College London	Moorfields Eye Hospital, University College London, UK	Wet AMD	hESC-RPE	Cell sheet—subretinal implantation	Sep-2012

Table 10.4 Recent and current transplantation studies (Device delivery approach)

Study ID	Study title	Phase	Sponsor/ collaborators	Location	Target disease	Cell source	Delivery approach	Registration date
NCT01949324	A Phase 2 Multicenter Randomized Clinical Trial of CNTF for MacTel	II	Neurotech Pharmaceuticals	Jules Stein Eye Institute, California, Bascom Palmer, Florida, Emory University, Georgia, National Eye Institute, Maryland, Massachusetts Eye and Ear Infirmary, University of Michigan, Retina Associates of Cleveland, Ohio, University of Wisconsin, US Centre for Eye Research, East Melbourne, Lions Eye Institute, Nedlands, Australia	MacTel (Type 2)	Human Cells releasing CNTF	ECT Intraocular Implant (NT-501)	Sep-2013
NCT01648452	CNTF Implants for CNGB3 Achromatopsia	I/II	National Eye Institute	National Institutes of Health Clinical Center, Maryland, US	Achromatopsia (CNGB3)	Intraocular Implant Releasing CNTF	Intraocular implantation	Jul-2012

NCT01530659	Retinal Imaging of Subjects Implanted With Ciliary Neurotrophic Factor (CNTF)-Releasing Encapsulated Cell Implant for Early-stage Retinitis Pigmentosa	II	Neurotech Pharmaceuticals, University of California	University of California, San Francisco, US	RP, Usher syndrome type 1, 2	Human Cells releasing CNTF	ECT intraocular implant (NT-501)	Jan-2012
NCT01327911	Ciliary Neurotrophic Factor (CNTF) Safety Trial in Patients With Macular Telangiectasia (Mactel)	I	Neurotech Pharmaceuticals, The Lowy Medical Research Institute Limited	Jules Stein Eye Institute, LA, California, Retina Associates of Cleveland, Ohio US	MacTel (Type 2)	Human Cells releasing CNTF	ECT Intraocular Implant (NT-501)	Jsn-2011
NTC00447954 NTC00447980 NTC00447993	A Study of Encapsulated Cell Technology (ECT) Implant for Participants With – Atrophic Macular Degeneration, – Late Stage Retinitis Pigmentosa, and – Early stage Retinitis Pigmentosa	II/III	Neurotech Pharmaceuticals	Retina-Vitreous Associates Medical Group, Beverly Hills California, Retina Group of Florida, Bascom Palmer Institute, Florida, Ophthalmic Consultants of Boston, Massachusetts, Beaumont Eye Institute, Michigan, University of Utah, US	Dry AMD Early RP Late RP	Human NTC-201 Cells Releasing Ciliary Neurotrophic Factor (CNTF)	Encapsulated Cell Technology (ECT) intraocular implant (NT-501)	Mar-2007

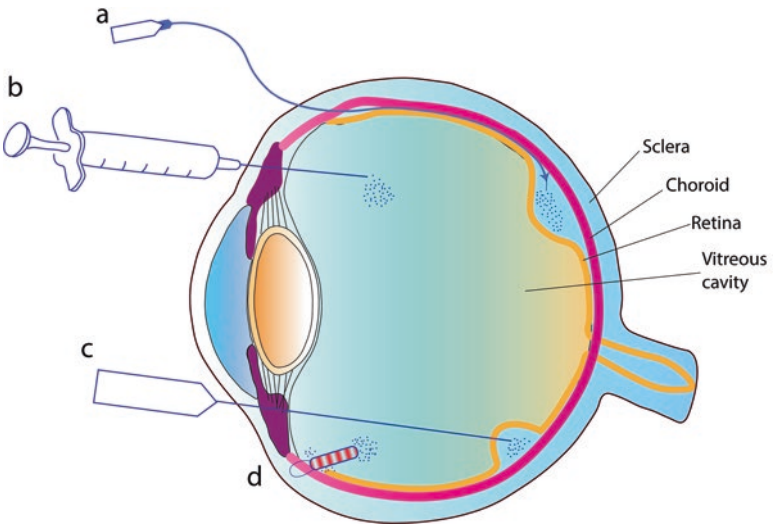

Fig. 10.1 Eye drawing illustrating the different access points and surgical approaches for therapeutic cell delivery. (**a**) Suprachoroidal approach: purpose designed microcatheter progressing through the potential space between retina and choroid, to inject the therapeutic cells into induced subretinal bleb. (**b**) Intravitreal approach: injection of the therapeutic cells directly into the vitreous cavity. (**c**) Transvitreal approach: injection of the therapeutic cells into the subretinal space via the vitreous cavity, after inducing a subretinal bleb with a small gauge cannula. (**d**) Cell device approach: intravitreal implantation and scleral fixation of therapeutic cell-loaded microdevice, which releases therapeutic factors into the vitreous cavity

In contrast to cell suspension delivery, transplantation of a cell sheet or patch requires a more complex surgical procedure. It necessitates a custom device capable of holding, protecting, and delivering the graft in a way that it sustains proper apical-basal orientation (assuming the cells are polarized) throughout its transplantation into the subretinal space. Furthermore, an adequately sized retinal incision is necessary for the sheet to be implanted. However, the benefits of this complex delivery procedure of sheet transplants are substantial, in terms of optimizing cell polarization, integration to the host tissues, and the potential size of the treated area.

Devices: Encapsulated Cell Technology (ECT)

ECT consists of a semipermeable polymer membrane capsule loaded with mammalian cells that have been genetically engineered to secrete therapeutic proteins.

Patented by Neurotech Pharmaceuticals, this novel drug delivery platform has offered an approach of overcoming the blood-retinal barrier, which—like the blood-brain barrier—restricts access of large molecules from the blood stream to the target cells. The circumvention of the blood-retina barrier is one of the major challenges for long-term sustained delivery of proteins to the retina for the treatment of a broad spectrum of eye diseases.

The semipermeable membrane of the ECT device allows the secreted protein to diffuse out and nutrients to diffuse in, but prevents access by the host immune system, thereby providing a sustainable supply of the therapeutic factor over an extended period, possibly years. In addition, the encapsulated cell implants can be retrieved from the eye at any time, providing an additional level of safety (Fig. 10.1d).

The most common therapeutic agents delivered by ECT are neurotrophic factors. These proteins can influence survival, proliferation, differentiation, and function of neurons and other cells in the nervous system and seem to hold a promising ability to retard progression of neurodegenerative disease. For the purpose of retinal neuroprotection the most studied protein is ciliary neurotrophic factor (CNTF).

It is anticipated that with further development of the ECT platform and similar approaches, future implants could become smaller and insertable in different locations, either anchored, free-floating in the vitreous cavity, or implanted subretinally, and will able to release specific proteins to replace proteins that are dysfunctional in retinal, RPE, and/or choroidal cells as a result of hereditary dystrophies.

Sites of Delivery and Current Methods of Access

It has been more than 30 years since the first description of RPE cell transplantation on to a denuded Bruch's membrane in owl monkeys, using an "open-sky" surgical technique and without attempt to reattach the retina [4]. During the last three decades, numerous transplantation techniques and cell delivery instrumentations have been introduced, a variety of which are being used currently in stem cell transplantation studies.

At present, the most broadly studied site for delivery of therapeutic cells is the subretinal space, i.e., the potential space between the neural retina and the RPE. Fewer trials are using the less complicated option of intravitreal delivery, while a very different "external" approach, which involves transscleral delivery and crossing the supra-choroidal space, has been applied for subretinal drug delivery and is now utilized for cell transplantation. Finally, future studies directed by tissue-specific treatment requirements may also focus on more accurate intraretinal and sub-RPE implantation.

Intravitreal

The intravitreal route delivers cells into the eye via injection using a small-gauge needle (Fig. 10.1b). Advantages of this method include technical simplicity and minimal invasion as it does not require a vitrectomy procedure. It can be performed in the office setting and it has been the most well studied and broadly used method for intraocular delivery of any therapeutic agent, since the advent of the anti-Vascular Endothelial Growth Factor (VEGF) injections for retinal diseases. It could be appropriate for the most prevalent diseases with high numbers of patients, such as AMD. This method,

however, also holds some significant disadvantages. First of all, it does not target the therapeutic cells directly to the degenerated tissue, and thus they have to migrate through the vitreous and retina in order to reach the outer retina or subretinal space. Transretinal migration has been shown for immune cells, RPE cells, and pigment granules {Burke:1982em} [5], while in terms of drug delivery, studies are confined to nanoparticles [6]. Another drawback of this approach is the exposure of the implanted cells in the vitreous to immune cells, such as macrophages. Transforming cells in the vitreous also have the potential to induce proliferative vitreoretinopathy and tractional retinal detachments. This potential risk has recently been accentuated by reports of severe retinal complications after intravitreal injections of experimental cell treatments [7–9].

Numerous researchers have adopted the intravitreal approach in both preclinical and clinical trials. Tracy et al. implanted bone marrow-derived mesenchymal stem cells (MSCs) from normal mice into the vitreous of mice undergoing retinal degeneration as a result of PPT1 gene mutation. The implanted cells showed survival without proliferating or invading the retina. This indicates that intravitreal implantation of MSCs is likely a safe means of long-term delivery of proteins synthesized by the implanted cells [10]. Park et al. conducted the first clinical trial in humans exploring the use of intravitreal autologous bone marrow CD34+ cells for ischemic and degenerative retinal disorders. Phase I outcomes reported feasibility and good tolerance which opened the field for further exploration [11].

Additionally, therapeutic approaches that involve factor-releasing cell devices, such as ECT for CNTF delivery, have been using the vitreous cavity as the implantation site for the device (Fig. 10.1d). The surgical procedure involves a small opening of the conjunctiva to access the sclera at the pars plana and a full thickness sclerotomy (approximately 2.5–3.0 mm) to access the vitreous cavity. The device is then inserted into the vitreous and anchored with scleral sutures. Finally, the sclerotomy is sutured and the insertion site is covered with re-apposition of the conjunctiva.

Preclinical studies using encapsulated cell-based CNTF delivery have offered evidence of photoreceptor protection in a dose-dependent manner when implanted into the eye of the *rcd1* dog with a cGMP-*PDE6b* mutation. The implants were seeded with human retinal pigment epithelium cells that had been transfected with the CNTF gene to produce CNTF protein in situ [12, 13]. Sieving et al. conducted a Phase I clinical trial of CNTF delivered by ECT in human subjects with advanced retinitis pigmentosa (RP). The planned follow-up period was 6 months in this initial study, after which the implants were surgically removed. No implant was rejected or extruded, and no severe systemic or ocular adverse events occurred. The investigators reported a trend to improved visual acuity in the study eyes [14]. Conversely, the results from a similar study by Birch et al. showed no efficacy of the CNTF against RP in the long term (60–96 months), while over the short term there were even signs of loss of visual field sensitivity of the treated eye, compared to the sham-treated eye. This loss was attributed to the active implant and was found reversible after its removal [15]. In another recent trial, patients with geographic atrophy (GA) associated with non-exudative late stage AMD received ECT implants anchored to the sclera in an anterior location in the vitreous cavity [16]. Although the trial failed to meet its primary endpoints, CNTF secretion persisted for up to 2 years [16]. More examples of trials using the intravitreal route are listed in Tables 10.2 and 10.4.

Subretinal

Both cell suspensions and cells-on-membrane sheet transplants have been targeted to this potential space, from which the new cells can interact and integrate with both the neural retina and the RPE/Bruch's/choriocapillaris complex. Due to this access, the subretinal delivery seems ideal for a large number of retinal degenerative diseases including AMD.

In most of the reported cell transplantation studies the subretinal space is accessed trans-vitreally. The procedure starts with a standard 23- or 25-gauge pars plana vitrectomy, followed by induction of a posterior neurosensory retina detachment using a stream of balanced salt solution via a small, usually 38–41-gauge cannula, in order to create a subretinal "bleb" of fluid. Subsequently, for a cell suspension implantation, another small (e.g., 38-gauge) cannula may be utilized for the subretinal injection through the same neuro-retinal puncture (Fig. 10.1c). In the case of cell sheet transplant, a larger retinotomy has to be performed in an extrafoveal location, through which the therapeutic patch is placed between the retina and the residual RPE, using a purpose-designed tool. Following inspection of the peripheral retina, a fluid-air exchange is performed, and, according to each study protocol, a tamponade agent is injected into the vitreous cavity (air, gas, or silicone oil). This approach has been tested in various therapeutic studies that have utilized stem cells as well as non-stem cell implantations.

Non-stem Cell Trials

Before the advent of stem cell-derived treatment in human trials, numerous researchers had attempted subretinal transplantation of either fetal or cadaveric tissue patches and/or suspensions, for the treatment of retinal degeneration.

Algvere et al. carried out a study of subretinal transplantation of human fetal RPE (13–20 weeks of gestational age) in patients with different forms of AMD. In one group, eyes with disciform lesions due to AMD underwent pars plana vitrectomy (PPV), excision of submacular fibrovascular membranes, and transplantation of a patch RPE transplant into the subretinal space. The patch (approximately $1.0 \times 1.5\ mm^2$) was initially sucked up into a purpose-designed glass micropipette (inner/outer diameters approx. 0.3/0.4 mm, respectively) filled with BSS and subsequently delivered into the submacular space through a retinotomy. In the second group, eyes with non-exudative AMD underwent PPV and peeling of epimacular vitreous membranes when needed, followed by the subretinal injection of a small patch-RPE transplant. The patch (0.6 mm diameter) was placed extrafoveally at the border of the GA area. In two other groups, patients with dry AMD and RPE tears respectively, were transplanted with a suspension of RPE cells through a small retinotomy, using a 20-gauge glass micropipette with a tapered tip (0.1 mm outer diameter), that had previously been flushed with sodium hyaluronate. The suspension was injected into the center of the macula. The retinotomy was small enough to self-seal and prevent the injected cells from refluxing into the vitreous cavity. In all groups the subretinal space was accessed

after inducing a neurosensory retinal detachment with a stream of fluid via a 33-gauge *Thomas* needle, so that a small bleb was created. The implantation/injection of the cells slightly enlarged the retinal bleb. The operation was completed with a fluid-air exchange and silicone oil tamponade for the first group and air-gas exchange (20% of SF6, or 12.5% of C3F8) and face-down posturing for 2-4 days for the other groups, where no subretinal tissue was removed [17]. After 24–38 months of follow-up, 12 of 16 grafts failed, and this was attributed to immune rejection. The risk of rejection seemed to be related to the integrity of the blood-retinal barrier (BRB) with both patch transplants and RPE suspensions being rejected early—within first 3 months—when placed over an exudative foveal area with compromised BRB. Nevertheless, allografts in non-exudative areas were lost more slowly—over 12–20 months—while extrafoveal transplants were retained after 30 months postoperatively.

A similar approach was reported by Kaplan et al. who describe two cases of transplantation of a sheet of human photoreceptor cells, harvested from cadaveric eyes, into two patients with retinitis pigmentosa. In this study the retinotomy was created with a myringotomy blade and extended with vertical scissors. The sheet of intact photoreceptors encased in gelatin was delivered subretinally through a pipette mounted on a specially designed delivery system. Subsequently, the subretinal bleb was partially flattened and fluid-gas exchanged was performed for pneumatic tamponade (20% SF6). Subjects did not receive any immunosuppression. There was no apparent rejection nor improvement in vision [18]. In contrast, when this group transplanted allogeneic RPE sheets into patients with exudative AMD (following choroidal new vessel excision), systemic immune suppression was required to maintain graft integrity [19].

Humayun et al. delivered a full-thickness undissociated sheet of fetal retinal tissue in the subretinal space of a patient with AMD, in addition to a microaggregate suspension of fetal retinal cells. The fetal neural retina was obtained from the optic vesicles of 14- to 16-week-old fetuses after scheduled pregnancy termination. Standard PPV and submacular surgery technique was used. A 2×2 mm^2 piece of retina was cut with microscissors and then grasped with a smooth-tip custom-built microforceps. The tip of the forceps was used to pierce the retina and, after entering the subretinal space, the tissue was released such that the outer retinal layer was facing the host RPE. Because there was an extensive disciform scar in the macula of the AMD patient, both the microaggregate suspension and the retinal sheet were transplanted in an extramacular location superior to the optic nerve head. No signs of rejection or visual improvement were shown [20].

Radtke et al. reported a case series of transplantation of fetal retinal sheets in patients with RP, and fetal retina together with its RPE in patients with advanced RP or AMD. For the delivery, a custom-made implantation instrument with a flat plastic nozzle tip at a 130-degree angle was used. The instrument maintained the orientation of the donor tissue. The loaded nozzle tip of the delivery instrument was inserted through the retinotomy into the submacular space, and the nozzle was released placing the retina/RPE sheet into the target area. The retinotomy was subsequently sealed by laser. No immunosuppression was given. Initially no signs of rejection nor improvement of vision was shown; however, in a follow-up publication, modest visual improvement was reported for 7 of the 10 patients [21, 22].

Stem Cell Trials

Transvitreal Access

In 2012 Scwarz et al. published the first description of a human stem cell-derived therapeutic trial for retinal degeneration. This was a phase I/II prospective study investigating safety in patients with advanced dry AMD or Stargardt disease. Subjects in the trial received a subretinal cell suspension of hESC-derived RPE (line MA09-hRPE). The operation followed the standard sequence: PPV, localized neuroretinal detachment, subretinal injection of the suspension in areas adjacent to GA loci, and finally air-fluid exchange. Systemic immunosuppression with Tacrolimus and mycophenolate mofetil was instituted for 12 weeks following the surgery. Schwartz et al. went on to publish their methods and the 18-month outcomes for 9 AMD patients and 9 Stargardt disease patients [23]. No serious ocular or systemic adverse events were reported. There was limited, pigmented, epiretinal membrane formation in some patients. Immune rejection was not recognized clinically. Areas of increased pigmentation at the transplantation sites were seen in 72% of subjects, while primary functional outcomes were reported to be promising. These results offered the first evidence of medium- to long-term safety, transplant survival, and possible function of pluripotent stem cell progeny in degenerative retinal disease [24]. Numerous current and recent studies have used similar methods and are listed in Table 10.2.

The subretinal space has also been used for SC-derived transplants in the form of a sheet [25, 26, 27]. Mandai et al. were the first to report the results of an induced pluripotent stem cell (iPSC)-derived RPE sheet transplantation in a patient with wet AMD. They demonstrated safety but no efficacy of their method, in terms of visual function [25]. The London Project (TLP) to Cure Blindness and University College London have commenced a Phase I study trying to reconstruct the anatomy of the subretinal space in severe wet AMD by implanting confluent, polarized hESC-derived RPE cells on an artificial basement membrane in the form of a "patch" [28]. This group uses submacular microsurgical techniques and a specially designed injector to insert the 6 × 3 mm lozenge-shaped patch into the subretinal space of patients who suffer from acute wet AMD with sudden severe vision loss due to submacular or sub-RPE hemorrhage or an RPE tear. For immunosuppression they use transient perioperative systemic steroids and, in the longer term, intraocular depot corticosteroid delivery devices. Two patients have received the patch so far, and the recently published one-year results were promising, with both patients having a significant improvement in visual acuity, reading speed, and retinal sensitivity [26]. Similar approaches utilizing cell sheet transplants are listed in Table 10.3.

Suprachoroidal Access

A completely different surgical method of accessing the subretinal space has been developed by Janssen (Titusville, NJ—division of Johnson and Johnson) in order to deliver human umbilical tissue cells (hUTCs) to patients with GA. These cells have been

evaluated in the Royal College of Surgeons rat model of retinal dystrophy and rescue degenerating photoreceptors better than other cell lines [29]. This technique utilizes a trans-scleral microcatheter-based delivery, which is advanced through the supra-choroidal space. The operation starts with a minor conjunctival dissection with surface cautery, followed by a scleral cut-down and a specialized scleral speculum insertion, 9 mm posterior to the limbus. The choroid is perforated, and a subretinal bleb is created with Healon®, under direct endoscopy (Endo Optiks, Little Silver, NJ). Subsequently, the 250-μm subretinal microcatheter (iScience Interventional, Menlo Park, CA) is inserted from the scleral opening and advanced through choroid into the subretinal space to the posterior pole (Fig. 10.1a). The tip of the catheter is illuminated and allows accurate localization to the areas of GA. The hUTC suspension is then injected by a high precision pump into the subretinal space. The catheter is carefully withdrawn, and all sclerotomies are closed with standard techniques. This surgical approach has still to be improved since some patients developed retinal tears and detachments.

Target Diseases and the Need for Specific Delivery Approaches

Retinal degenerative diseases constitute a large, heterogeneous group of inherited or acquired disorders that disturb mainly the photoreceptor and the RPE layers, the function of which constitute the most critical layers for visual function of the eye.

AMD

Age-related macular degeneration is associated with a chronic, low-grade inflammation that affects the outer layers of the central retina, starting with the degeneration of the RPE and Bruch's membrane and leading to loss of photoreceptors and subsequent Geographic Atrophy (GA). GA is expected to affect 3.8 million adults by the year 2050 [30]. Even patients with the neovascular type of the disease (wet AMD) that can be stabilized using anti-VEGF injection treatments eventually manifest dry AMD. Furthermore, although anti-VEGF treatment may delay the progression of disease, there are significant drawbacks both for the patients, regarding the duration of therapy and the risk of complications, and for the health systems, regarding the financial burden of treating constantly increasing numbers of patients.

For a surgical AMD treatment to be feasible, it has to be technically simple, with low risk of complications, applicable in an office-based ophthalmological therapeutic setting, relatively inexpensive, and suitable for large numbers of patients. Cell-based treatments, trying to replace the RPE or RPE-Bruch's complex with stem cell-derived equivalents, hold promise for the future but face many challenges in terms of delivering a viable therapeutic option on a large scale. Numerous approaches have been tried so far, with most common among them being the subretinal injection of stem cell-derived RPE cell suspensions (Table 10.2), while most recent human studies of RPE-artificial BM sheets transplanted subretinally are yet to prove their feasibility and efficacy (Table 10.3).

Inherited Retinal Disease

In addition to the epidemiological and clinical significance of AMD, the management of AMD using a cellular approach also constitutes a potential therapeutic paradigm for other disorders that affect RPE and neural retina, such as inherited retinal diseases (IRDs).

Retinitis pigmentosa (RP) is the most prevalent of the IRDs affecting approximately 1/4000 individuals [2]. It is associated with primary photoreceptor degeneration due, in most cases, to defective genes involved in their metabolism. Several studies mainly using human retinal progenitor cells or human bone marrow mesenchymal stem cells to rescue or replace the degenerating photoreceptors are now running as shown in Table 10.2. Furthermore, some subtypes of RP caused by RPE-specific genetic defects seem to primarily disturb the structure and function of this supportive epithelial layer. Dystrophies associated primarily with the RPE specific genes such as MERTK [31] and RPE65 [32] could also be potential targets for cell-based RPE therapies in the future.

Stargardt disease is a juvenile retinal dystrophy caused by a photoreceptor gene defect that is associated with increased production of toxic bisretinoids and which leads to abnormal RPE lipofuscin accumulation and secondary RPE degeneration. Classically, it presents during the first two decades of age, and it is the commonest cause of juvenile macular disease, reducing central vision in approximately 1:10000 young individuals [3]. The first cell-based therapeutic study directed at Stargardt disease attempted to replace defective RPE by subretinal injection of a stem cell-derived RPE cell suspension. The results of this trial have offered the first long-term safety evidence and also suggested potential vision and vision-related quality of life improvement.

Other IRD examples that may be treated using cell-based therapies in the future include diseases such as vitelliform dystrophy (Best disease), choroideremia, cone and/or rod dystrophies, and some forms of Leber congenital amaurosis. It is also possible that retinal disorders with breaks to Bruch's membrane and secondary RPE atrophy, such as angioid streaks and myopia, may be amenable to an artificial membrane strengthening Bruch's with RPE cell replacement to reduce the effect of the secondary atrophy.

Uveitis: "Cellular Immunotherapy"

Apart from acquired and inherited retinal degeneration, cell-derived treatments have also been directed towards modifying other disease processes such as inflammatory ocular diseases. *Cellular immunotherapy* is an approach that uses intact, fully differentiated, autologous or allogeneic mature immune cells to modulate the patient's inflammatory reaction against a specific hazard.

More specifically, cellular immunotherapy is already being studied as a treatment of CMV retinitis that typically occurs in immunocompromised patients with insufficient primary T-cell response against the virus. In this approach, partially matched donor CMV-specific cytotoxic T-cells are infused intravenously into patients with CMV retinitis who are resistant, refractory, or intolerant to conventional antiviral therapies. Primary results have demonstrated efficacy against persistent viremia or systemic infection in the stem cell transplant population [33, 34].

Discussion

Feasibility Criteria

For a surgical approach to be adopted in everyday clinical practice, it has to meet some feasibility criteria. First of all, it has to demonstrate adequate safety for both the target tissue—the retina—and the adjacent tissues. The risk of complications such as retinal hemorrhage, retinal perforation, retinal detachment, and choroidal hemorrhage has to be comparable with other already established procedures. Secondly, the cell delivery approach must secure not only the initial implantation, but also the retention of the therapeutic cells in the targeted location. Leakage of cells either into the vitreous or in the suprachoroidal space may not only compromise the treatment, but also put the patient at risk, in case of cell migration to distant organs.

Additional requirements concern the procedural complexity and efficacy. The targeted delivery has to be reproducible, with straightforward adoption by experienced surgeons. Ideally, it is compatible with commonly used surgical tools and techniques and has a duration suitable for high patient numbers. Ideally, the approach should also be adaptable to differing eye length and globe volume and expandable to be applied in a variable spectrum of cases.

Future Directions

The emerging progress in multimodal medical imaging and surgical instrumentation technology will open numerous new fields in therapeutic delivery in ophthalmology. Intraoperative OCT (optical coherence tomography) systems, already in use, and 3D surgical visualization systems are now in the process of changing the way ophthalmologists perceive eye surgery. The ability to obtain and analyze scans in real time as well as the option to superimpose simultaneous and/or previous exams onto the surgeon's view of the operational field in real time will soon provide an upgraded level of microscopic interaction between the surgeon and the target tissues.

Future developments in ophthalmological surgery, instrumentation, and robotics engineering are expected to overcome the challenge of insufficient surgical dexterity. Micro-precision devices such as surgeon extenders and teleoperated robots coupled with multimodal imaging sourced information will augment the effectiveness of eye surgeons in accessing and manipulating retinal and subretinal tissues. Targeting specific layers and microscopic structures within the retina in an accurate and safe manner may open delivery approaches that are not feasible at present [35]. In the near future, intraretinal, intra-choroidal, and intra-optic nerve cell treatments are expected to extend to currently untreatable diseases, the powerful new paradigm of cellular therapy for the treatment of an increasing number of blinding ocular degenerative diseases.

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Chapter 11
Diagnosis and Complementary Examinations

Young Ju Lew and Jacque L. Duncan

Introduction

Measures of visual function provide information about how the patient experiences the world and therefore provide clinically meaningful outcome measures of disease severity and progression in patients with retinal degenerative disease. For this reason, regulatory agencies such as the United States Food and Drug Administration (FDA) value tests of visual function as outcome measures of disease severity. Unfortunately, some of the most commonly used visual function tests, including visual acuity, are insensitive monitors of disease severity or progression, while more sensitive tests of visual function are often variable in patients with retinal degenerative disease. This chapter outlines measures of visual function and retinal structure that are used commonly to diagnose patients with retinal degenerative disease and to monitor disease severity during disease progression and in response to experimental therapies. In addition, a Clinical Statement on the American Academy of Ophthalmology website (https://www.aao.org/clinical-statement/recommendations-on-clinical-assessment-of-patients) summarizes a standard approach to the evaluation and management of patients with inherited retinal degenerations, which can supplement this chapter.

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Visual Function Testing

Visual Acuity

Retinal degenerative diseases have various clinical features depending on the cell type primarily affected. Therefore, visual acuity alone may not be a sensitive measure of disease progression. However, visual acuity is the most widely used measure of visual function and remains a basic method to assess changes from early to late stages of disease progression. An acuity chart based on Sloan letters was developed for the Early Treatment Diabetic Retinopathy Study (ETDRS) [1], and the ETDRS chart has served as the standard measure of visual acuity in clinical trials. However, in conditions such as retinitis pigmentosa (RP), central visual acuity is maintained until late stages of the disease (including the stage at which the patient is legally blind due to severe loss of peripheral visual field (<20°). Furthermore, as visual acuity declines, patients find it increasingly difficult to use the foveal cones to look at objects, or maintain foveal fixation, and rely increasingly on retinal locations outside the fovea, or extrafoveal fixation, which is often unsteady. Steady fixation is required to measure visual acuity reliably. For these reasons and because clinical trials must demonstrate a change of at least 3 lines, or 15 letters, on the ETDRS chart before the change is considered significant by the FDA [2], visual acuity is an insensitive measure of disease progression in many retinal degenerations.

The ETDRS chart has five letters per line and 0.1 log mean angle of resolution (MAR) progression from line to line, so each correct letter is worth 0.1/5 = 0.02 logMAR. Although differences between scoring methods are usually small, it has been shown that letter-by-letter scoring is more reproducible than line-by-line scoring [3, 4]. ETDRS charts are available for test distances ranging from 4 m (13 ft.) to 2 m (6.5 ft.), and when used at the designated distance, they can measure acuity from 20/10 to 20/200. Given the logarithmic progression of letter size, however, they can be used at any distance with appropriate correction of the results [5].

If patients cannot read any letters, they are moved closer to the chart or are measured with an electronic version of the visual acuity measurement protocol called Electronic-ETDRS Visual Acuity (EVA). EVA has been developed to more easily standardize measurement of visual acuity in clinical trials and to provide a method to directly capture acuity data electronically [6]. EVA utilizes a personal computer and a high-resolution monitor to present letters of standardized luminance (85–105 cd/m^2) and contrast (90%) [6]. EVA testing is performed at 3 m rather than the standard 4 m used with standard ETDRS charts, and letters are presented individually with crowding lines [6, 7]. EVA testing has been accepted for use as a primary clinical trial endpoint by the FDA for clinical trials [7].

Visual Field

Retinal degenerations involving rod photoreceptors typically involve photoreceptors throughout the retina, and usually spare the central vision until late in the disease due to the high density of cones in the fovea. By contrast, in cone dystrophies, cone-rod dystrophies, and macular dystrophies such as Best disease, central vision is degraded before peripheral vision. In patients with typical RP (i.e., rod- or rod-cone dystrophy), visual acuity tends to be an insensitive measure of disease severity, and other tests of visual function are required to diagnose retinal degenerations in their early stages, while sensitive tests of macular function are necessary to diagnose macular, cone, and cone-rod dystrophies in their earliest stages. Two types of perimetry, static automated perimetry and kinetic perimetry, are widely used by many clinics. In static automated perimetry, a stationary target is changed in size and brightness until seen, while in kinetic perimetry a target of predefined size and luminance is moved from a non-seeing to a potentially seeing area.

Kinetic perimetry is particularly useful for monitoring peripheral visual field defects, large scotomata, and the progression of visual field loss over time because it tests the visual field to 90° temporally. Several studies have characterized and compared distinct patterns of visual field progression with specific genetic mutations in an attempt to better understand the subgroups of RP [8, 9]. Quantitative analysis of structural measures of the outer retinal layers using spectral domain optical coherence tomography (SD-OCT) to assess disease progression has shown that visual recognition of the I4e isopter correlates with retinal areas showing an intact inner segment ellipsoid zone [10]. For these reasons, kinetic perimetry traditionally has been the standard measure for observing and documenting progression of retinal degenerations, including RP. Kinetic perimetry has limitations, however, including inter-test variability of up to 50% in RP patients [11, 12], but test-retest variability of functional areas identified with kinetic perimetry in RP can be limited to <20% by using a single experienced operator [13].

In retinal degenerations, static perimetry can be used to assess macular sensitivity where macular dystrophy (e.g., Stargardt disease or pattern dystrophies) or cone-rod degeneration is suspected. Automated perimetry correlates with contrast sensitivity and is a sensitive predictor for central visual function in advanced RP [14, 15]. More recently, algorithms have been created to interpolate the hill of vision measured by full-field static perimetry using a technique known as visual field modeling and analysis [16]. Rather than measuring average sensitivity across the field, which may be insensitive to local scotomata, the hill of vision provides a topographic endpoint that quantifies global and local sensitivity. The hill of vision analysis provides a quantitative, reliable measure of visual field that has been validated in clinical trials [17–19] and correlated with structural measures [20], and is therefore a commonly used measure of visual function for clinical trials in retinal degenerations.

The relationship between the functional change reflected in visual field sensitivity and anatomical change reflected in the ellipsoid zone and outer nuclear layer thickness measured using OCT has been studied [21, 22]. For the rate of decline in visual

field sensitivity in X-linked RP, the location just inside and outside the edge of the ellipsoid zone degeneration marks a transition zone between relatively healthy and relatively degenerate retina that is the most dynamic region during disease progression [23].

Microperimetry

Microperimetry, or fundus-related perimetry, is a technique that involves static or kinetic visual field testing while observing the retina simultaneously to track eye motion using either scanning laser ophthalmoscopy (SLO) or an infrared fundus camera [24]. Eye-tracking provides high-accuracy functional measures, even in cases of unstable or extrafoveal fixation [25]. In RP, retinal sensitivity measured with microperimetry shows a significant correlation with total retinal thickness and outer retinal thickness measured with SD-OCT [26], and microperimetry values change in parallel with structural changes measured using SD-OCT in early age-related macular degeneration over a 12-month period [27]. Fundus-related microperimetry is very useful in patients with central scotomata or eccentric fixation and may be a helpful measure of macular function in patients with Stargardt disease [28] and other retinal degenerations that affect the macula. Limitations of microperimetry include significant test-retest variation in patients with choroideremia, ceiling effect in individuals with preserved macular function, and limited dynamic range, which makes testing difficult in patients with advanced degeneration [29, 30].

Full-Field Stimulus Test (FST) and Light- and Dark-Adapted Perimetry

Psychophysical tests to determine rod- and cone-based visual function in different retinal regions have been developed to understand different disease patterns across the retina [31]. The Full-field Stimulus Test (FST) uses a brief full-field (Ganzfeld) flash and provides a luminance threshold [32, 33]. After subjects are fully dark-adapted, short- and long-wavelength stimuli are delivered using a full-field flash, and the difference in sensitivity to the two stimuli can be used to determine whether the thresholds are rod-mediated, cone-mediated, or a combination of both at the most sensitive regions of the retina. Although the stimuli used in FST and full-field electroretinogram (ERG) are similar, the FST is a threshold response that is assumed to be mediated by the most sensitive cells of the retina, whereas full-field ERG reflects integrated responses from the entire retina [34].

The FST has been found to be useful in patients with severe retinal degeneration, such as Leber congenital amaurosis, RP, and cone-rod dystrophy, in which patients have difficulty maintaining steady fixation [34–36]. Furthermore, the FST has been found to correlate with full-field ERG in Stargardt disease and RP [32, 35]. The FST

could be a useful psychophysical test for assessing cone and rod function in patients who are not able to perform a visual field test or who have non-detectable ERG responses, and in clinical trials that have focal targets (i.e., subretinal gene or stem cell delivery). However, FST measures visual function from the most sensitive retinal areas without localizing the region responsible.

Automated perimeters can be used to measure and compare light- and dark-adapted sensitivity to provide localized measures of rod- and cone-mediated function throughout the visual field [31]. Although this approach does not control for unstable fixation, it provides regional measures of rod- and cone-mediated function throughout the visual field, which facilitates identification of retinal regions with preserved photoreceptors prior to delivery of localized treatments, such as subretinal gene therapy, and measurement of treatment response in those regions.

Electrodiagnostic Tests

Electroretinogram (ERG)

The full-field ERG plays an important role in the diagnosis and characterization of inherited retinal degenerations by providing a global assessment of photoreceptor function. ERG changes can identify the type and extent of photoreceptor dysfunction, often prior to funduscopically detectable changes, and can be used to monitor disease progression over time.

The ERG represents a mass electrical response generated by light-induced changes in extracellular electrolytes (mainly Na^{2+} and K^{+}) at the level of the photoreceptor outer segments [37]. The ERG is recorded under photopic (light-adapted) and scotopic (dark-adapted) conditions, and the International Society for Clinical Electrophysiology of Vision (ISCEV) standards specify six responses based on the adaptation state of the eye and the flash strength: (1) Dark-adapted 0.01 ERG (rod ERG); (2) Dark-adapted 3 ERG (combined rod-cone standard flash ERG); (3) Dark-adapted 3 oscillatory potentials; (4) Dark-adapted 10 ERG (strong flash ERG); (5) Light-adapted 3 ERG (standard flash "cone" ERG); and (6) Light-adapted 30 Hz flicker ERG (a sensitive cone-pathway-driven response) [38]. The a-wave (Fig. 11.1a) is cornea-negative and originates from a light-induced hyperpolarization of the rod and cone photoreceptor outer segments [37]. The a-wave is best measured in response to a bright flash (3.0 cd s m^{-2}) in dark-adapted (scotopic) and light-adapted (photopic) conditions [38]. The b-wave is cornea-positive and occurs after the a-wave (Fig. 11.1a). It is a consequence of bipolar cell depolarization and provides information on post-photoreceptor signal transduction. Wavelets on the upslope of the b-wave are called oscillatory potentials and likely indicate activity of horizontal and amacrine cells [39]. Retinal degenerations primarily affect the photoreceptors and, therefore, cause a reduction in both the a- and b-wave responses from rods or cones primarily. Disorders that cause dysfunction of post-photoreceptor transduction can selectively reduce the b-wave, a finding known as a negative ERG waveform (Fig. 11.1d, e) [37].

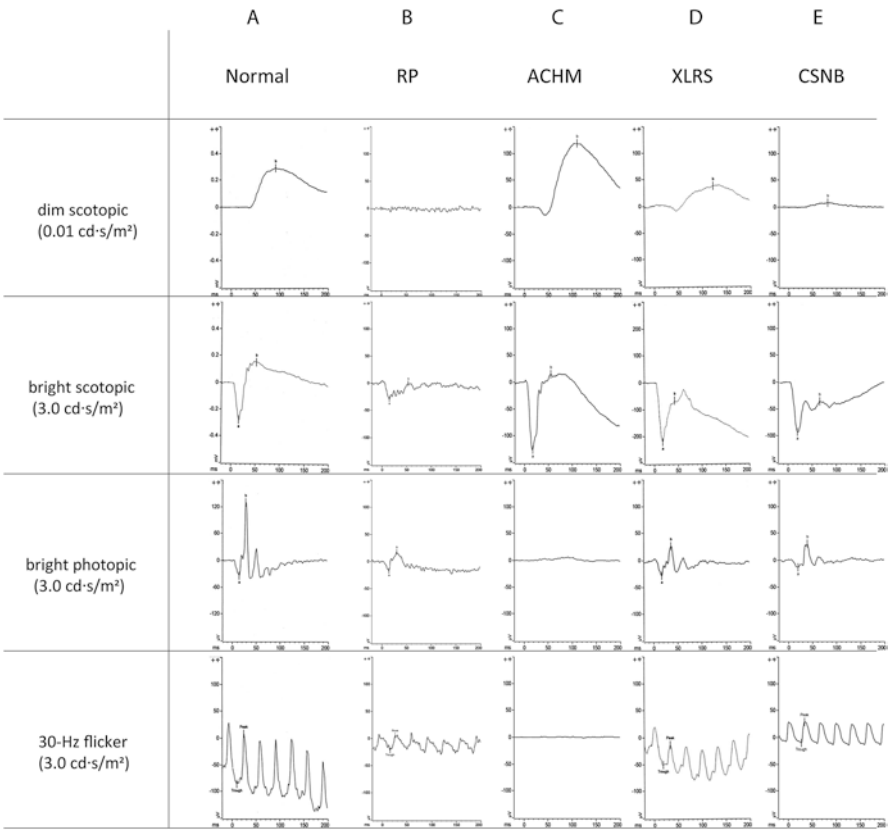

Fig. 11.1 Full-field ERG responses recorded according to ISCEV standards [38] (**a**) Representative normal ERG responses. (**b**) ERG from a patient with retinitis pigmentosa (RP) reveals severe reduction in amplitude of the rod and mixed cone-rod responses, while the cone responses (bright photopic and 30-Hz flicker) are moderately reduced. (**c**) ERG from a patient with congenital achromatopsia (ACHM) showing near-normal scotopic responses and non-recordable cone responses. (**d** and **e**) Negative ERG waveforms with a preponderance of second-order neuron dysfunction in (**d**) a patient with X-linked retinoschisis showing a severely reduced b-wave in the scotopic responses, and (**e**) a patient with complete congenital stationary night blindness (CSNB) of the Schubert-Bornschein type with a non-recordable b-wave in the scotopic responses. Hatch marks indicate trough of a-wave and peak of b-waves

According to the ERG responses, retinal degenerations may be characterized as causing rod-cone, cone-rod, or second-order neuron dysfunction. RP is a typical example of rod-cone degeneration. ERG responses in RP patients can range from normal to non-detectable according to their genotype and age, but when responses are obtainable, rod responses are typically affected to a greater extent than cone responses (Fig. 11.1b). The amplitude of the responses is proportional to the area of functioning retina [40].

Cone-rod disorders are divided into diseases that are congenital with very slow progression, such as achromatopsia and blue cone monochromatism, and those that typically show normal function at birth with more marked progressive dysfunction, such as cone dystrophy and cone-rod degeneration [41]. The typical ERG finding of the congenital condition achromatopsia is an absent or nearly absent cone response with delayed bright flash scotopic responses and with normal or near-normal rod-mediated responses (Fig. 11.1c). In cone dystrophy or cone-rod degeneration, the often subtle or absent funduscopic changes make the ERG an important tool in the diagnosis of a cone dystrophy. Rod responses are usually normal or only minimally affected in the early stages. With time rod and cone responses show progressive amplitude reduction [41].

In patients with second-order neuron dysfunction (post-photoreceptor transduction), selective reduction of the b-wave can occur in the presence of preserved a-wave photoreceptor responses. When the b-wave to a-wave ratio is equal to or smaller than 1, a "negative ERG" is present [42]. Two disease entities that may be associated with a negative ERG are X-linked retinoschisis (XLRS) and congenital stationary night blindness (CSNB) (Fig. 11.1d, e, respectively).

The ERG has value both in the diagnosis and monitoring of disease progression in RP and other inherited retinal degenerations. It is estimated that, on average, patients with RP lose about 16–18.5% per year of remaining ERG amplitudes [12, 43]. The ERG can provide an objective measure of retinal function in response to new therapies such as gene replacement, stem cell therapy, or neuroprotective factors in animal studies. However, the ERG is insensitive to changes in macular function and subtle changes in photoreceptor survival because it is a pan-retinal response, and in many patients with advanced retinal degeneration the full-field ERG is not detectable, although visual acuity and visual field may remain measurable.

Multifocal Electroretinogram

The multifocal ERG (mfERG) permits assessment of cone diseases affecting local regions of the retina. The mfERG is normally recorded under photopic conditions and employs 103 hexagons flickering at a rate of 75 Hz, eliciting cone-mediated responses [44]. The hexagons are scaled with eccentricity such that each hexagon represents a response recorded from a similar number of cones. The magnitude of the responses is calculated as the response density, representing response amplitude per unit retinal area [44]. The mfERG responses represent retinal function from the central 40–50° of the macula corresponding with automated perimetry tests of the central macula, and the mfERG can be used to identify regional outer retinal dysfunction. Each waveform of the first-order response consists of an initial negative deflection (N1) followed by a positive peak (P1), followed by a second negative deflection (N2). The cellular origin of the major negative and positive waves is similar to the full-field ERG, but the mfERG technique uses a pseudorandom m-sequence that correlates global electrical responses from the retina with

the regions that were stimulated to identify localized retinal responses throughout the central macula [45]. Thus, despite the similarities between the mfERG and the full-field ERG, the mfERG responses are not "small ERGs" [44].

The mfERG can be used to detect functional loss in the central retina before funduscopic changes occur. A clinical application of mfERG includes the assessment of retinal toxicity associated with systemic medications, such as chloroquine and hydroxychloroquine, vigabatrin, ethambutol, canthaxanthine, and tamoxifen [46]. Besides being a useful tool in screening for toxic maculopathies, mfERG helps to establish the diagnosis in macular dystrophies and diseases characterized by interocular and intraretinal variation, such as occult macular dystrophy, white dot syndromes, and acute zonal occult outer retinopathy (AZOOR) [47]. The result is typically combined with automated perimetry spanning the central 60° of visual field and microstructural changes of OCT in order to establish the correct diagnosis. Furthermore, the correlation of the mfERG with other measures of visual function, such as visual acuity and full-field ERG, has been studied in patients with RP; mfERG amplitude and visual field sensitivity correlated well with SD-OCT outer retinal thickness, but the mfERG amplitude and visual field sensitivity were more sensitive indices of photoreceptor degeneration than SD-OCT receptor layer thickness in the fovea [48]. In addition, intact inner/outer segment junction or ellipsoid zone lines in SD-OCT images correlated with better visual field sensitivity and mfERG amplitudes [49]. The combined use of mfERG and these tests may offer a sensitive and useful method for determining therapeutic efficacy in treatment trials for RP or other diseases.

Electro-oculogram

The electro-oculogram (EOG) measures an electrical potential, known as the standing potential, of about 6 mV between the anterior and the posterior regions of the eye, which changes during dark- and light-adapted conditions [37]. The standing potential is an indirect measure of the trans-epithelial potential (TEP) of the retinal pigment epithelium (RPE), and the TEP is equal to the difference in the membrane potential of the basolateral and the apical membranes, which are electrically isolated through the tight junctions of the RPE [50]. The light peak:dark trough (LP:DT) ratio is an index (Arden ratio) used to assess retinal function, and this ratio is $\geq$1.8 in normal eyes. Because the LP:DT ratio of the EOG is affected in some diffuse disorders of the RPE and the photoreceptor layer, EOG abnormalities are proportional to the severity of rod-mediated ERG abnormalities [50]. In conditions that primarily affect RPE cells, however, such as retinal degeneration associated with mutations in the bestrophin gene (*BEST1*), the EOG may be selectively abnormal in the presence of a normal full-field ERG (Fig. 11.2). Bestrophinopathies include Best vitelliform macular dystrophy (BVMD), autosomal recessive bestrophinopathy (ARB), and autosomal dominant vitreoretinochoroidopathy (ADVIRC). Best vitelliform macular dystrophy is characterized by abnormal EOG in the

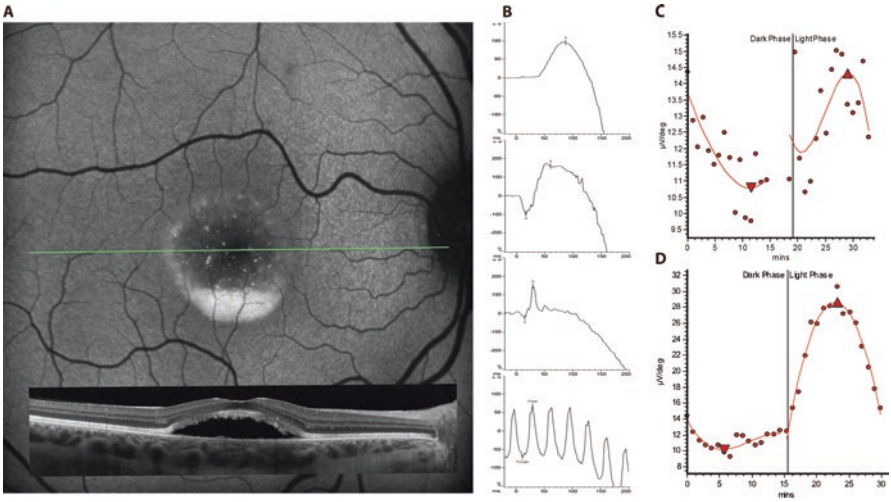

Fig. 11.2 Clinical characteristics in Best macular dystrophy. (**a**) Comparison of fundus autofluorescence photograph and SD-OCT image of the right eye of a 28-year-old male with Best macular dystrophy associated with a heterozygous mutation in the *BEST1* gene (c.652 C > T, p. Arg218Cys). The fundus autofluorescence image illustrates the vitelliform pseudohypopyon lesion, while the SD-OCT B-scan image demonstrates hypo-reflective subretinal fluid associated with the vitelliform pseudohypopyon lesion. (**b**) Full-field ERG shows normal scotopic and photopic responses. (**c**) Abnormal EOG showing a reduced light peak to dark trough ratio (Arden ratio = 1.33). (**d**) Normal EOG (Arden ratio = 2.82). Note the y-axis scales are different in (**c**) and (**d**). Hatch marks indicate trough of a-wave and peak of b-waves

context of normal full-field ERG responses, which may allow detection of asymptomatic carriers. However, a normal EOG does not exclude the possibility of BVMD since approximately one-third of patients in a reported series had a *BEST1* mutation with a normal EOG [51]. In ARB and ADVIRC, the ERG is often abnormal, but the light peak of the EOG is nearly absent, which indicates that RPE function is more severely or disproportionately abnormal in comparison to photoreceptor dysfunction [52–54]. In combination with ERG and mfERG, EOG provides useful information about the integrity of photoreceptor and RPE cell function.

Imaging Technology

Structural Measures

Optical Coherence Tomography (OCT)

By providing noninvasive, cross-sectional images of the retina, OCT has revolutionized the way ophthalmologists evaluate retinal diseases and treat patients. Furthermore, OCT has been regarded as one of the most objective, reliable, quick,

and precise test methods used to measure retinal structure in clinical trials. The introduction of SD-OCT improved image resolution and scan speed with speeds ranging from 29,000 to 80,000 scans per second and axial resolution up to 2 μm in commercially available systems [55, 56]. The enhanced depth image (EDI) mode was developed to improve the visualization of deep structures, such as the outer retina, RPE, choroid, and sclera [57]. Swept source OCT (SS-OCT) uses a short cavity swept laser with a tunable wavelength of operation instead of the superluminescent diode laser used in SD-OCT [58]. SS-OCT has improved image penetration using a wavelength of 1050 nm, with an axial resolution of 5.3 μm and axial scan rate of 100,000 scans per second [56]. The 12 × 9 mm scan enables simultaneous imaging of the macula, the peripapillary area, the optic nerve head, and the choroidal thickness [59]. SS-OCT images in a normal subject show varying reflective properties of the different retinal layers and four distinct bands in the outer retina (Fig. 11.3a, b) [60–62].

The thickness of the outer retinal layers in the transition zone between healthy and severely affected retina may provide a sensitive measure of disease progression [63]. The earliest change in the transition zone seen in OCT images is a decrease in the thickness of the outer segment (OS) layer, followed by decreases in the inner segment (IS) layer and ONL [63–65]. The point at which the OS region disappears correlates with the location at which visual sensitivity shows precipitous changes [22]. The OS thickness is measured between the EZ band and the proximal border of the RPE. Thus, when the EZ band disappears (i.e., is no longer discernible from the RPE border), the OS thickness is zero [23]. Decreased ONL thickness and loss of the EZ band have been associated with visual field defects and provide an easily obtainable measure of disease progression and response to therapy [10, 22]. For the rate of decline in visual field sensitivity in X-linked RP, the transition zone has been found to be the most dynamic region during disease progression [23]. Recently, the EZ width, EZ area, EZ transition zone, and ONL thickness have been accepted as structural biomarkers in RP [20, 23, 66], while the external limiting membrane (ELM) provides a measure of photoreceptor inner segment integrity and may be an important marker of photoreceptors in which the outer segments have degenerated, but the photoreceptor nuclei and inner segments persist in eyes with advancing retinal degeneration [67].

Fundus Autofluorescence (FAF)

Fundus autofluorescence (FAF) is a noninvasive imaging technique used to visualize naturally or pathologically occurring fluorophores that absorb and emit light of specified wavelengths. Clinically significant fundus fluorophores include lipofuscin bisretinoids [68] that are present in RPE cells and may increase in eyes with drusen in age-related macular degeneration, *ABCA4, BEST1,* and *RDS*-related retinal degenerations and in optic disc drusen [69]. Commercially available FAF systems include fundus cameras, confocal scanning laser ophthalmoscopes (cSLO), and ultra-wide field technologies [69]. The fundus camera is a digital system that captures AF using

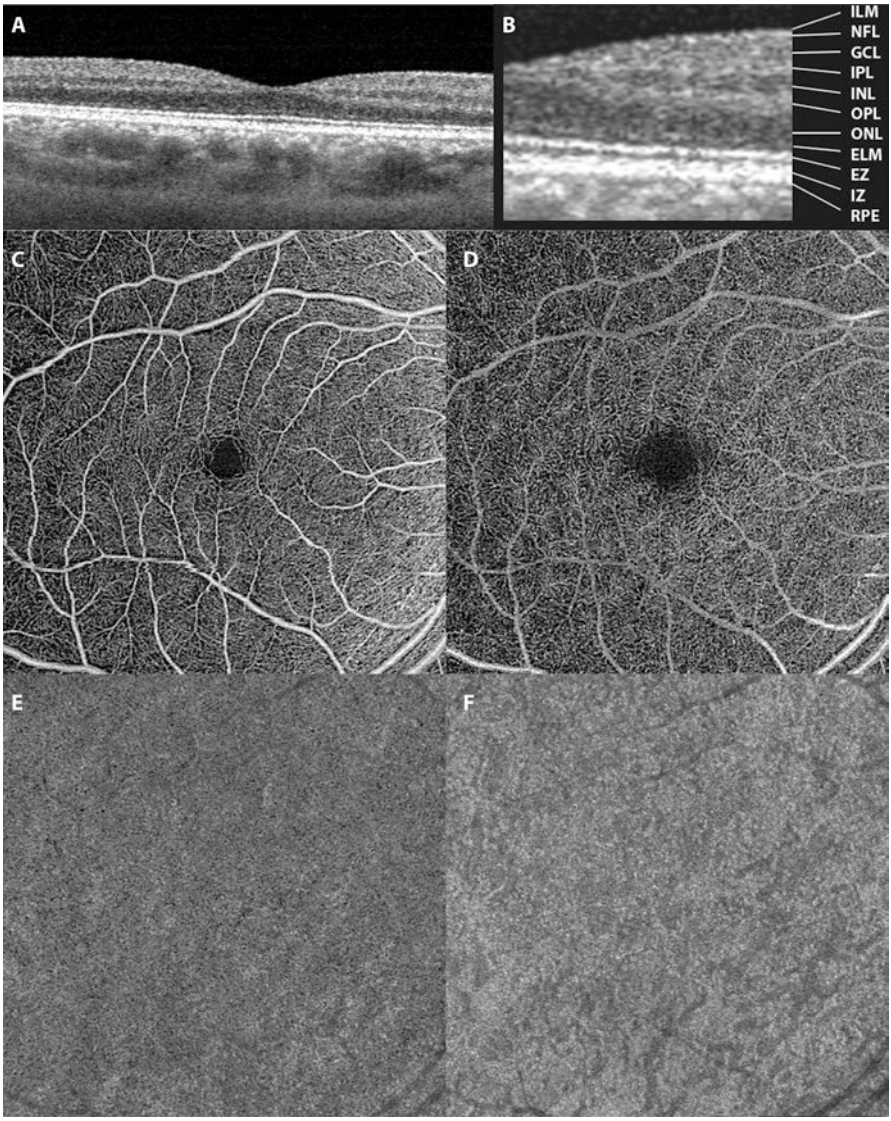

Fig. 11.3 Swept Source OCT (SS-OCT) images (Everest PLEX Elite OCTA, Carl Zeiss Meditech, Dublin, CA) in a 24-year-old normal subject. A horizontal B-scan through the fovea shows varying reflective properties of the different retinal layers (**a**), labeled in (**b**). The first hyper-reflective band at the vitreoretinal interface is the internal limiting membrane (ILM), followed by the hyper-reflective nerve fiber layer (NFL) and by a less hyper-reflective band composed of the ganglion cell layer (GCL). The inner plexiform layer (IPL) is hyper-reflective. The hypo-reflective inner and outer nuclear layers (INL and ONL) are separated by the hyper-reflective outer plexiform layer (OPL). The SD-OCT typically resolves 4 distinct hyper-reflective bands in the outer retina; the innermost band is the external limiting membrane (ELM), followed by the ellipsoid zone of the photoreceptor outer segments (EZ), the interdigitation zone between the RPE apical processes and the photoreceptor outer segments (IZ), and the outermost band composed of the retinal pigment epithelium plus a possible contribution of Bruch's membrane and choriocapillaris to the hyper-reflective band (RPE). OCT angiography provides *en face* images of superficial retinal capillary (**c**), deep retinal capillary (**d**), choriocapillaris (**e**), and choroidal (**f**) vascular perfusion

a single flash of light with an excitation spectrum of 535–580 nm and a 615–715 nm emission barrier filter, which results in reducing AF of the lens and cornea [70]. Fundus cameras demonstrate higher rates of successful image acquisition in patients with cataract and also provide better detection of exudative retinal disease, such as choroidal neovascularization and central serous chorioretinopathy compared to cSLO systems [71, 72]. However, the absence of confocal optics makes the fundus camera prone to light scattering and generation of secondary reflectance light that interferes with FAF detection [69]. The cSLO utilizes a system of mirrors to focus a low-power laser in a two-dimensional raster pattern onto the fundus [69]. The cSLO normally uses a blue excitation wavelength of 488 nm and an emission filter between 500 and 700 nm [73]. The cSLO has advantages such as decreased scattered light and real-time averaging, which offers high-contrast and high-resolution images [74]. However, the cSLO has limitations. The excitation beam is absorbed by macular pigment, and thus cSLO autofluorescent images cannot be obtained after fluorescein angiography [75]. Ultra-wide field SLO fundus imaging systems simultaneously use two excitation wavelengths of red (633 nm) and green (532 nm) light with an emission filter of >540 nm [69]. Ultra-wide field imaging permits imaging the peripheral fundus, including up to 82.5% of retinal surface area, but peripheral images may be distorted [69].

In maculopathies associated with mutations in the *RDS* gene, AF images can show more widespread abnormalities than are visible on color fundus photos (Fig. 11.4a, b). In Stargardt disease, lipofuscin accumulation is seen in fundus "flecks" (drusen-like structures) that are associated with an intense, focally increased AF signal. AF images sometimes reveal areas of atrophy (hypofluorescent areas) or flecks (hyperfluorescent areas) not seen on color fundus photography, emphasizing their potential for early disease detection (Fig. 11.4c, d) [76]. FAF has been correlated with visual function in Stargardt disease, with normal macular AF associated with normal electroretinography findings and good vision [77]. In patients with RP, the hyper-autofluorescent ring, which corresponds to the border of inner/outer segment junction disruption and lipofuscin production, has been shown to correlate with preserved central photopic function and visual field sensitivity within the ring [78, 79]. Serial imaging of this hyper-autofluorescent ring may provide information about stability or rate of progression of the disease in RP.

Vascular Perfusion

Fluorescein and Indocyanine Green Angiography

Fluorescein angiography (FA) uses intravenous sodium fluorescein, an orange-red crystalline hydrocarbon that is excited by 465–490 nm blue light and emits 520–530 nm green-yellow light. Sodium fluorescein, which is water soluble, easily diffuses through the fenestrated vessels of the choriocapillaris but does not pass through normal retinal vascular endothelium and RPE tight junctions, which comprise the inner and outer blood-retinal barriers, respectively [80]. The interpretation

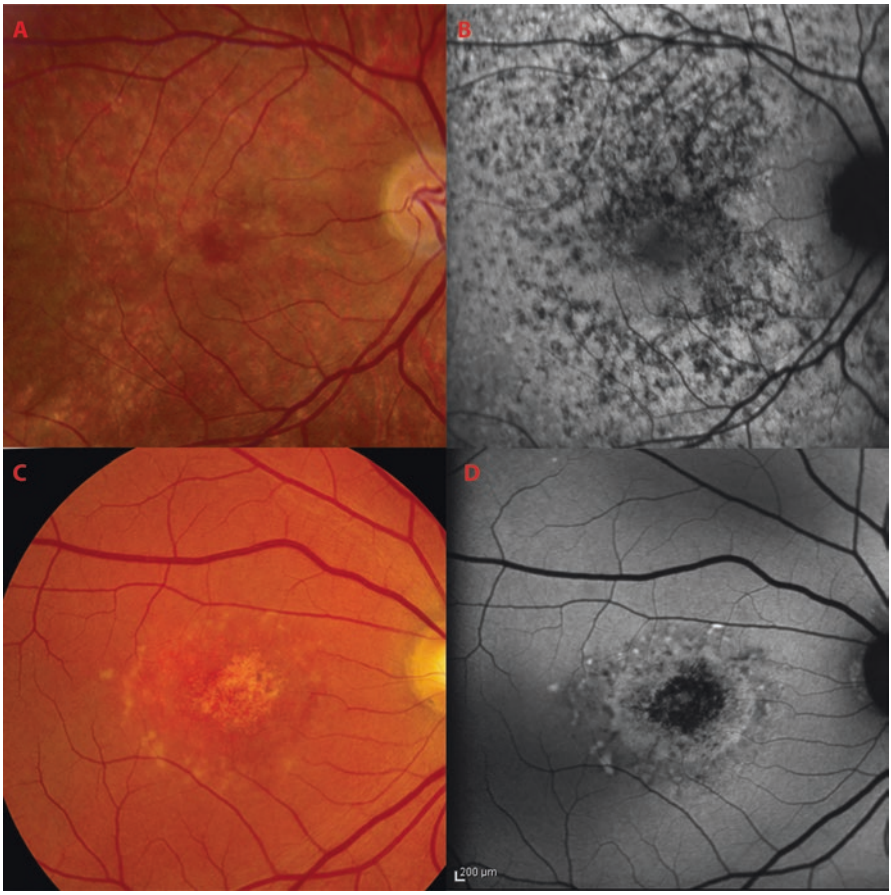

Fig. 11.4 Fundus autofluorescence images in retinal degenerations. (**a**) Color fundus and (**b**) fundus autofluorescence images taken from a 45-year-old woman with a heterozygous mutation in the *RDS* gene (c. 637 C > A, p. Cys213Tyr); visual acuity was 20/20. (**c**) Color fundus and (**d**) fundus autofluorescence images taken from a 55-year-old woman with compound heterozygous mutations in the *ABCA4* gene (c. 634 C > T, Arg 212 Cys; c. 2589 G > C, p. Gly 863 Ala; c. 3265 C > T, p. Thr 959 Ile); visual acuity was 20/150. Note the different SW-FAF signals of the flecks in panel **d** with some revealing a focally increased SW-FAF whereas others present with decreased SW-FAF, perhaps due to a loss of RPE cells heralding a progression to atrophy. Short-wavelength fundus autofluorescence (SW-FAF) images were taken using a blue excitation (488 nm) in each patient

of the FA follows a simple and logical progression according to abnormal fluorescence (i.e., hyperfluorescence or hypofluorescence), location, transit time, and leakage [80]. These factors provide insight into vascular abnormalities and help identify potentially treatable sequelae of retinal diseases, such as breakdown of the outer blood-retinal barrier [80]. Abnormalities of fluorescence transmission may be seen in patients with Stargardt disease, who demonstrate a dark choroid due to a blockage of choroidal fluorescence by lipofuscin accumulation within RPE cells. However, the use of FA has continued to decline because of concerns of

short-wavelength light exposure and the risk of phototoxicity in eyes with retinal degeneration, because animal models have shown increased damage to photoreceptors after exposure to short-wavelength light [81].

Indocyanine green angiography (ICGA) allows visualization of the dye through overlying melanin, xanthophyll pigment, hemorrhage, and lipid exudates. ICG absorbs light at 790 nm (near-infrared) and emits at approximately 835 nm. The ICG is an anionic tricarbocyanine dye with a high molecular weight and high plasma protein binding (up to 98%) [82]. These properties reduce the amount of dye that exits from choroidal vessel fenestrations. ICG can be used to visualize choroidal neovascularization, central serous chorioretinopathy, choroidal tumors such as choroidal hemangioma and choroidal melanoma, and choroidal inflammation. It can, therefore, be a valuable tool when dealing with occult macular pathologies lacking biomicroscopic changes [83, 84].

Optical Coherence Tomography Angiography (OCT-A)

The FA and ICGA are both invasive techniques that require intravenous injections of dye to obtain high contrast images of the retinal circulation. Moreover, the FA and ICGA are unable to evaluate the blood flow or vascular density in each vascular plexus. OCT-A provides a novel method for noninvasively imaging the capillary network and the foveal avascular zone in discrete retinal layers (Fig. 11.3c–f) [85]. OCT-A uses amplitude or phase decorrelation technology with high-frequency and dense volumetric scanning to detect blood flow and to visualize blood vessels at various depth-resolved levels of the retina and choroid [86].

Microvascular structures such as vascular densities and avascular zone areas have been studied in normal eyes (Fig. 11.3) and in various retinal diseases using OCT-A [87–89]. Vascular densities of the superficial and deep retinal capillary plexus were significantly decreased in RP patients compared to normal subjects, and OCT-A provides a sensitive measure of preserved choroidal and choriocapillaris vasculature in eyes with choroideremia [90, 91].

Adaptive Optics (AO) Imaging

Flood-Illuminated Adaptive Optics (AO)

The main barrier limiting resolution of all standard clinical images of the retina is uncorrected aberrations in the light exiting the eye, which are introduced by subtle irregularities in the optical media. Adaptive optics (AO) uses a wavefront sensor to measure the ocular aberrations and compensates for them with a deformable mirror, generating noninvasive, high-resolution images of the retina [92, 93]. AO compensates for optical aberrations in the eye's optics and can be applied to any ophthalmoscope modality, including full-field fundus camera, cSLO, and OCT

systems [94]. A commercially available AO flood-illuminated imaging system (Imagine Eyes, Orsay, France) uses a conventional fundus (flood illumination) camera [95]. The camera consists of three subsystems: (1) adaptive optics for compensation of the eye's wave aberrations, (2) pupil retro-illumination and a fixation channel for alignment of the subject's eye to camera, and (3) retinal imaging using a novel fiber-based light source and scientific-grade charge coupled device (CCD) [95].

The correlation between FAF, SD-OCT, and waveguiding cone structures visualized with flood-illuminated AO in RP has been studied [96, 97]. Cone density varied with different sampling methods and regions tested in normal eyes, such that large changes in cone density are required to reliably demonstrate disease progression and that further repeatability and sampling studies are needed [98]. Although the commercially available flood-illuminated AO may not offer high resolution images of cones within about 1° of the fovea, it can provide images of melanocytes and pigment within RPE cells, which may provide useful measures of disease progression in age-related macular degeneration [99], and flood-illuminated AO images may be useful when interpreted in the context of other imaging modalities such as SD-OCT and FAF.

Adaptive Optics Scanning Laser Ophthalmoscopy (AO-SLO) Confocal Images

The most significant achievement of AO-assisted imaging is the observation of waveguiding structures in living human eyes, and this is main advantage of AO-SLO images acquired using a confocal SLO configuration [92, 94]. Confocal AO-SLO images demonstrate retinal microstructures that are waveguiding, such as the nerve fiber layer, photoreceptors, retinal pigment epithelial cells, and retinal vasculature, with higher resolution than is possible with flood-illuminated systems [94]. The SLO forms an image over time by continually recording the light scattered from a single focused spot as the light is scanned across the retina, generally in a raster pattern [94]. The confocal imaging technique is achieved by placing a spatial pinhole at retinal conjugate plane to include only direct backscattered light, while multiple scattered light (out-of-focus) is simultaneously eliminated. The transverse resolution is approximately 2 μm, permitting visualization of individual photoreceptors (Fig. 11.5). In healthy eyes, cones appear as bright spots arranged in a hexagonal pattern with regular spacing (Fig. 11.5b), while in eyes with retinal dystrophies cones can show abnormal morphology, including spacing and packing patterns with increased cone spacing and sparse cone mosaics in regions with extensive cone loss (Fig. 11.5d) [100–103]. Changes in cone spacing, or average distance to the nearest neighboring cone, and cone density have been reported in various retinal diseases and have been used to monitor cone structure during disease progression and to assess the effect of ciliary neurotrophic factor (CNTF) in patients with inherited retinal degenerations [104]. AO-SLO images demonstrated a significant difference in the rate of progression measured both by cone spacing and cone density between CNTF- and sham-treated eyes, while traditional

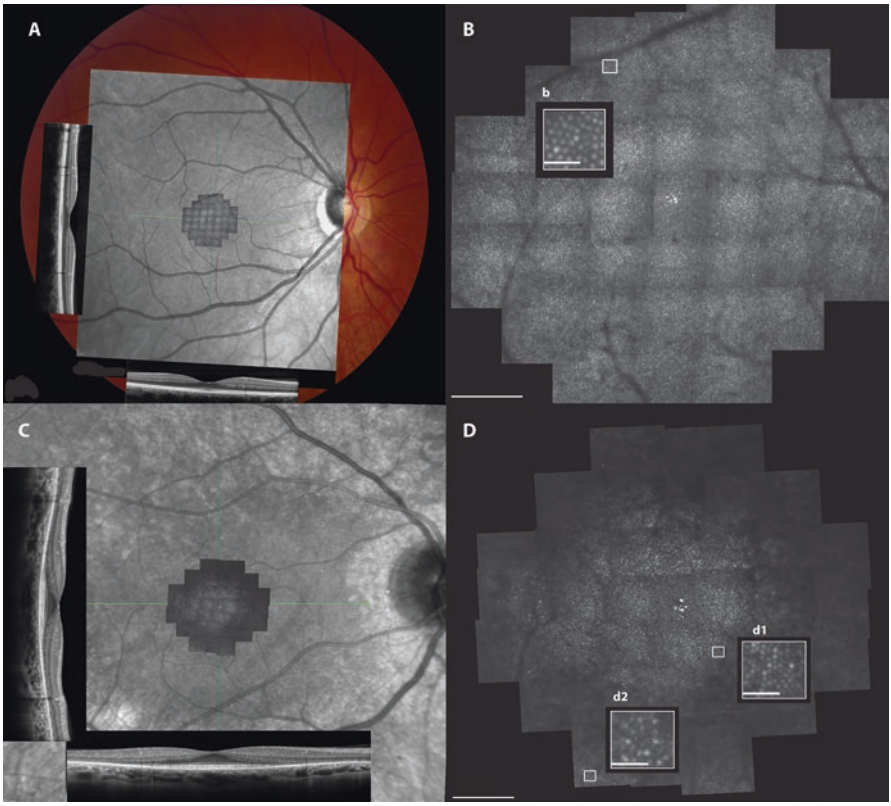

Fig. 11.5 Confocal adaptive optics scanning laser ophthalmoscopy (AO-SLO) image from a normal 44-year-old subject (**a**) and a 39-year-old patient with simplex RP (**c**) superimposed on color fundus photo and infrared fundus photos using retinal vascular landmarks to precisely align images from multiple modalities. Vertical and horizontal sectional SD-OCT images are shown with green lines on the infrared fundus photo to indicate the location of the scan in each figure. Figures (**b**) and (**d**) show AO-SLO montages with magnified insets showing cone photoreceptors as white spots in regular mosaics in a normal eye (b), and in a region with well-preserved cones in a mosaic in an eye with RP (d1). Other regions in the montage from the eye with RP show bright spots that do not correspond to cones (d2) because they are not arranged in hexagonal mosaics, and there is no inner segment/outer segment band on the OCT scan corresponding to that region. Larger scale bar at the bottom is 1° and the magnified inset extends 0.1° on each side

outcome measures such as visual acuity, visual field sensitivity, and ERG showed no significant difference [104]. However, confocal AO-SLO images provide information about waveguiding structures in the retina, and the mosaics in AO-SLO images include only cones with intact IS/OS junctions and OS/RPE junctions. When photoreceptor OSs are disrupted, they no longer waveguide normally and are often not visible in confocal AO-SLO images [105]. Finally, images of structures distal to the IS/OS junction are not visualized in eyes with intact photoreceptor mosaics using near-infrared

light because the light waveguided by the inner segment-outer segment junction precludes visualization of light from structures that lie external to it. Investigators have used short-wavelength light and ICG to visualize RPE cells in eyes with intact photoreceptors [106, 107], and 2-photon microscopy to visualize Muller cells, ganglion cells, and inner retinal cells [108], but these approaches present a potential risk of phototoxicity and have not been used in humans.

Adaptive Optics Scanning Laser Ophthalmoscopy (AO-SLO) Nonconfocal Images

In confocal imaging systems, the confocal pinhole blocks scattered light except that which comes from near the plane of focus. However, nonconfocal AO-SLO systems have been developed to image scattered light that is captured with a displaced pinhole. When the pinhole is displaced, the confocal light is blocked, and only nonconfocally scattered light can reach the detector [94, 109]. The confocal pinhole has been replaced by different nonconfocal detection schemes, the split-detector showing the most widely used result by revealing the vessel and capillary walls with the most detail and contrast across all retinal locations and depths [110]. Split-detection AO-SLO systems can collect confocal, dark-field, and nonconfocal images simultaneously [111, 112]. Using this split-detection technique, photoreceptor inner segments can be seen in a manner that appears to be independent of the integrity of the outer segment, and in patients with achromatopsia, cone inner segments occupied the majority of the dark gaps in the confocal AO-SLO images [105, 109, 113–115]. The RPE cell mosaic was imaged using nonconfocal detection "dark-field" AO-SLO by attenuating the light backscattered by the photoreceptors in a healthy subject [112]. Using combined confocal and nonconfocal split-detector system, clusters and mosaics of photoreceptors and possible macrophages can be seen within the retinal lesion in various stages of Best vitelliform macular dystrophy [116], suggesting that evaluating split-detection and dark-field AO-SLO images in the context of confocal AO-SLO and OCT images is likely to provide the most meaningful interpretations of retinal and RPE cell structure.

Adaptive Optics Optical Coherence Tomography (AO-OCT)

While OCT provides subcellular resolution in the axial direction, its transverse resolution is limited [111]. Thus, the combination of AO with OCT has improved the transverse resolution of 3- dimensional retinal cellular structures. AO-OCT has allowed for 3-dimensional imaging of cone and rod photoreceptors and visualization of cone outer segment tips, retinal nerve fiber bundles, lamina cribrosa, and retinal vasculature. The addition of AO to commercial OCT and ultrahigh-resolution OCT improves their resolution volume by 36 times and achieves a resolution voxel smaller than most retinal cells.

Conclusion

Photoreceptors, the cells that initiate visual perception, are the primary site of disease in patients with retinal degenerations, so accurate assessment of visual function and retinal structure is critical for correct diagnosis and management of patients with retinal degenerations. Novel measures of visual function provide new ways to monitor the impact of different diseases on rods and cones, and advances in retinal imaging permit high-resolution, objective ways to evaluate retinal structure. As research developments provide insight into disease mechanisms that may lead to new treatments for patients with retinal degenerations, accurate diagnosis and measurement of disease progression will become essential for: (1) designing and evaluating the results of clinical trials and (2) managing patients once treatments are available. Multimodal approaches that correlate and combine measures of visual function and retinal structure will provide the most precise characterization of disease phenotype and will enable reliable and sensitive measures of disease progression in patients with retinal degenerations. For newer, high-resolution imaging modalities to serve as outcome measures for clinical trials, it is necessary to validate the inter-visit and inter-grader variability of quantitative measures as well as correlation with retinal function [115, 117]. The use of higher-resolution imaging results may accelerate the pace at which treatments for retinal degenerations are developed.

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Chapter 12
Cell Therapy for Degenerative Retinal Disease: Special Focus on Cell Fusion-Mediated Regeneration

Francesco Sottile, Martina Pesaresi, Giacoma Simonte, and Maria Pia Cosma

Degenerative Retinal Diseases

Degenerative retinal diseases represent a heterogeneous group of conditions ascribable to a variety of different causes and generally leading to severe visual disability and blindness. Retinal neurons strictly communicate through an elaborate net of connections; therefore, any weakness in these networks could potentially lead to retinal disease, retinal causes of blindness, Age-related macular degeneration (AMD) is the most frequently recorded cause of retinal blindness, followed by glaucoma and diabetic retinopathy [1]. AMD is a complex disease with a polygenic hereditary component [2, 3], is the leading cause of blindness in industrialized countries, and is estimated to affect 196 million worldwide by 2020 [4, 5]. AMD can be described as a progressive chronic disease leading to degeneration of the retinal pigment epithelial (RPE) and photoreceptor (PR) cells [6].

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Two distinct, but not mutually exclusive, forms of AMD have been described, namely exudative (or wet) and non-exudative (or dry) [7]. The former is characterized by abnormal choroidal neovascularization leading to hemorrhage, subretinal scarring, and PR death; it can be managed reasonably well with anti-angiogenic (anti-VEGF) drugs, which can stop, or at least delay, vision loss in a substantial majority of patients [8–10]. The latter, instead, is associated with geographic atrophy (i.e., apoptosis of PRs RPE, and subjacent choriocapillaris). Geographic atrophy progresses slowly but is currently incurable.

Abnormal retinoid processing due to a mutation in PRs with secondary RPE lipofuscin accumulation, and RPE and PR death occurs in Stargardt disease (STGD). STGD is associated with central visual loss, and is the most common inherited macular degeneration in children. It affects approximately 1 in 10,000 individuals worldwide[11, 12].

Retinitis Pigmentosa (RP) is a group of retinal dystrophies, all associated with a progressive and severe loss of rod and cone photoreceptors. Generally, vision loss begins in childhood with night blindness (nyctalopia), followed by loss of mid-peripheral visual field, and, eventually, loss of central vision and blindness [13, 14]. The disease has a prevalence of 1 in 4000 people, with over 100 causative genetic mutations identified. The vast majority of the reported mutations affect photoreceptors directly, even if some RP subtypes are associated with primary defects in the RPE [14].

Finally, optic neuropathies are a heterogeneous group of conditions associated with optic nerve damage. This damage can be due to trauma, infection, noninfectious inflammation including autoimmune disease (e.g., neuromyelitis optica), ischemia, or glaucoma. Currently, there is no reliable treatment for the vast majority of optic neuropathies, even if some of them can be managed with surgery, corticosteroids, immunosuppressants, and/or other drugs [15, 16].

Tissue Regeneration

The term regeneration describes the restoration process that is initiated in order to reestablish, at least partially, the physiological and morphological functions of damaged tissues or organs. During evolution, three main regeneration mechanisms have been selected. The first mechanism, named epimorphism, involves dedifferentiation of residing cells that form a specialized structure called a blastema; in turn, blastema cells re-differentiate, generating the new tissue [17]. This regenerative process has been observed during limb reconstitution in different organisms, including frog, newt, and salamander [18].

Alternatively, differentiated cells in proximity of the injured site can reenter the cell cycle, proliferate and generate identical daughter cells that are dedicated to the regeneration of the damaged tissue. This process is also referred to as “compensatory regeneration” and, despite the absence of dedifferentiation steps, it requires major gene expression changes for the reentry into the cell cycle [19]. Mammalian

liver regeneration is a classic example of compensatory regeneration, as it involves the enlargement of intact tissue to restore physiological function [20, 21].

Importantly, mammalian tissues contain a "reservoir" of adult stem cells (ASCs, also called somatic stem cells) that are crucial for both normal tissue homeostasis and repair after injury [22]. Generally, ASCs are located within a specialized tissue niche, where they are maintained in a quiescent state [23]. However, if exposed to the appropriate stimuli, ASCs can become active, leaving their niche and differentiating into tissue-specific cell types in order to replace deteriorated cells and maintain organ integrity [24].

Importantly, this endogenous repair mechanism can modestly rescue tissue functionality following damage although it appears quite inadequate in the face of severe injuries such as myocardial infraction and cerebral ischemia.

Nevertheless, recent advances in ASC isolation and manipulation are allowing us to gain an increasingly deeper and more complete understanding of the molecular mechanisms that regulate their regenerative capabilities. This information, in turn, is facilitating the refinement of related cell therapy approaches [25, 26]. Similarly, the use of pluripotent cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to replace damaged cells and repair injured tissues have been expanded.

In this chapter, we will briefly discuss the potential sources of stem cells used for treatment of retinal disease, with particular emphasis on both the beneficial and the deleterious effects of stem cell-based therapy.

Stem Cell Therapy for Retinal Disease Treatment

The high genetic heterogeneity associated with retinal diseases and the lack of effective therapies have invigorated the use of stem cell therapy approaches, aimed at either rescuing or replacing damaged cells in order to restore retinal functionality (Table 12.1).

The eye represents an exceptionally good target for CNS cell therapy, for a variety of reasons. First, surgical techniques for intravitreal and subretinal transplantation are already well established and routinely performed clinically. Additionally, a good number of noninvasive and high-resolution ocular imaging techniques are available, introducing the possibility of closely monitoring patients and the viability of transplanted cells; these techniques include optical coherence tomography (OCT), fluorescein angiography (FA), and adaptive optics scanning laser ophthalmoscopy (AO-SLO) [27–31].

Furthermore, the eye is a small and encapsulated organ, with visual acuity depending only on a relatively small number of cells. In other words, transplants of about 250,000 surviving cells would probably be sufficient to ensure a reasonable life standard that includes reading and possibly even driving.

Last, but not least, the subretinal space can be regarded as a relatively immune privileged site, at least under normal, non-disease circumstances [32, 33]. The RPE

Table 12.1 Comprehensive list of stem cell-based studies in rodent models of retinal degeneration and disease (*S* subretinal, *I* intravitreal)

Cell type	Disease	Route	Model	References
MSCs	Retinal degeneration	S	Rat (RCS)	[216, 234] [207]
			Rat (Light-damage)	[235]
	RP	S	Mouse (Rhodopsin KO)	[95]
	Glaucoma	I	Rat (Laser-induced ocular hypertensive glaucoma)	[236]
			Rat (Ligation of episcelar veins)	[205]
	Trauma	I	Rat (Optic Nerve Transection)	[103, 237]
BMDCs	Retinal degeneration	S	Rat (RCS)	[94]
		I	Mouse ($Pde6b^{Rd1}$ and $Pde6b^{Rd10}$)	[118]
	RP	S	Mouse ($Pde6b^{Rd10}$)	[238]
		I	Mouse (NMDA-induced RGC degeneration)	[197]
	Trauma	I	Mice (Laser injury)	[239]
	Retinal vasculopathy	I	Mice (Acute retinal ischemia-reperfusion injury)	[240]
ESC/iPSC-derived RPE	Retinal degeneration	S	RCS Rat	[56, 61, 241] [60]
	STGD	S	Elov14 Mouse	[61]
RPE	Retinal degeneration	S	RCS rat	[242]
Neural Progenitor Cells	Retinal degeneration	S	RCS rat	[42, 43] [45]
		I	Mouse (*mnd* mouse—lysosomal storage disease with retinal and CNS degeneration)	[41]
	RP		Mouse ($Pde6b^{Rd1}$ and $Pde6b^{Rd10}$)	[46]
	Trauma		Rat (Mechanical injury)	[50]
	Ischemia		Rat (Damaged by acute ocular hypertension)	[48]

(continued)

Table 12.1 (continued)

Cell type	Disease	Route	Model	References
RPCs	RP	S	Mice (Rhodopsin KO)	[220]
			Rat (Mutations in the rhodopsin gene)	[219]
Photoreceptor Precursors	RP	S	Mouse (Rhodopsin KO)	[58]
	Congenital stationary night blindness		Mouse ($Gnat1^{-/-}$, lacking rod function)	[79]
hMGC-derived RGCs	RGC degeneration	I	Rat (NMDA-induced RGC degeneration)	[87]

cells help to establish this immune privilege by: (1) inhibiting T-cell activation [34]; (2) secreting other immunosuppressive factors [35]; and (3) inducing conversion of CD8+/CD4+ cytotoxic T lymphocytes into regulatory T cells [36, 37]. This immune privilege reduces the chances of immune rejection of allogeneic cell therapy by the recipient's immune system.

Sources of Stem Cells for Retinal Disease Treatment: The Use of Pluripotent Stem Cells

A critical aspect of cell-based therapy involves identification of an appropriate stem cell source. In fact, the number of potential sources for therapy in retinal diseases is strikingly high. Among them, pluripotent stem cells such as embryonic stem cells (ESCs) probably hold the biggest potential for cell replacement strategies, and for diseases like RP and AMD.

ESCs are pluripotent cells derived from the inner cells mass (ICM) of the early blastocyst. They are able to generate an entire new organism [38], and, in vitro, they can be differentiated into any cell type, excepting those of the extraembryonic tissues (e.g., the placenta). Since the establishment of reliable cell culture conditions for ESCs (in the early 1980s), numerous studies have demonstrated their prominent differentiation capability [39, 40]. This ability is being exploited for the development of cell replacement strategies, based on in vitro ESC differentiation prior to transplantation in vivo.

For example, neural stem cells [41–43] and retinal progenitor cells [44] have been derived from both mouse and human ESCs and then transplanted into degenerating retinae. However, the number of studies investigating the beneficial effects of neural precursor cells (NPCs) in the eye is quite limited. Nevertheless, there is evidence of transplantation being beneficial in various models of retinal degeneration [42, 45, 46]. These beneficial effects have been attributed to the phagocytic activity of NPCs,

which can effectively delay photoreceptor degeneration [47]. However, NPCs do not seem to be a suitable option for the treatment of optic neuropathies [48], and, despite being capable of successful integration, they can differentiate neither into mature retinal ganglion cells (RGCs) nor into photoreceptors (PRs) [49, 50].

Although ESCs have been successfully differentiated in vitro into RPE cells [51] and PRs [52, 53], the integration efficiency of these newly generated cells into the neural circuit remains low [41, 54–57].

The use of ESCs for clinical purposes also needs to face additional problems, including: limited availability, ethical concerns, transplanted cell rejection by the recipient's immune system, and the high cost of the differentiation procedures. Additionally, ESC/iPSC-based cell therapy can raise controversial concerns with respect to the risk of tumorigenesis [58]. In one report, for example, transplanted ESC-derived neural precursors generated teratomata in 50% of the treated animals within 8 weeks from engraftment [59]. However, an increasing number of studies indicates that malignant transformation might be a side effect associated with transplant of multipotent cells; terminally differentiated ESC-derived cells do not seem to pose a threat with respect to tumor formation [58, 60]. Indeed, Lu and colleagues transplanted terminally differentiated ESC-derived cells into 45 immunodeficient mice lacking both mature T and B cells: none developed teratomata throughout their life [61].

In contrast to ESCs, iPSCs offer the attractive possibility of autologous transplantation (Fig. 12.1). In fact, they can be derived from reprogramming of the patient's

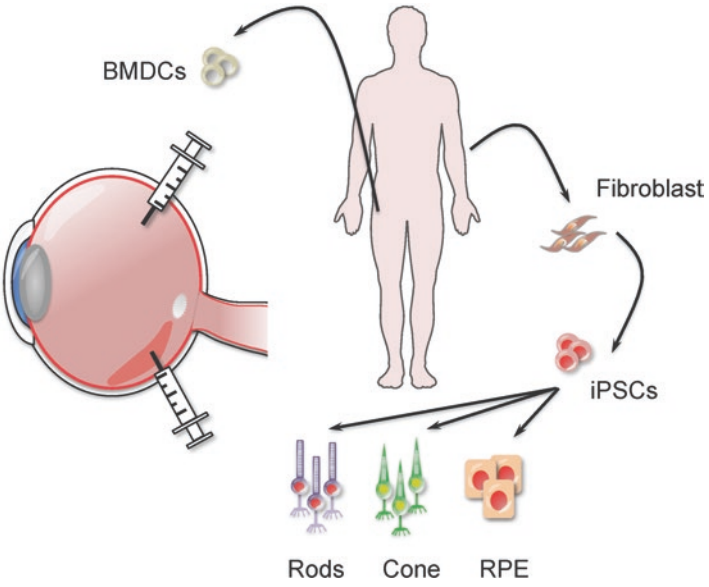

Fig. 12.1 Stem cell therapy for retinal regeneration. Induced pluripotent stem cells (iPSCs) derived from patient's somatic cells (e.g., fibroblasts) and bone marrow-derived cells (BMDCs) represent promising sources for the generation of new retinal neurons with the aim of treating degenerative retinal diseases. BMDCs include hematopoietic stem and progenitor cells (HSPCs) and mesenchymal stem cells (MSCs) that can be used for autologous transplantation

own somatic cells. This process involves the conversion of a fully differentiated cell back to a pluripotent state [62]. In other words, the use of patient-specific iPSCs should drastically reduce the risk of rejection by the recipient's immune system [62, 63]. Nonetheless, still there are considerable concerns regarding their potential immunogenicity [64, 65]. This may be due to some abnormalities in gene expression of iPSC-derived cells, which would be sufficient to induce T cell-dependent immune response even in syngeneic recipients [64]. Notably, compared to ESCs, iPSCs can show significant differences with respect to gene expression and DNA methylation patterns [66]. Indeed, human iPSC-derived RPE show a gene expression profile that significantly differ from that of human fetal RPE, whereas that of ESC-derived RPE is much more similar to the endogenous one [67].

The use of iPSCs also faces challenges in the context of genetic diseases. In fact, iPSCs derived from the patients will still harbor the disease-causing mutation, and, in most cases, genetic correction would be required prior to transplantation. Additionally, iPSCs have been traditionally associated with safety concerns deriving from the use of viruses to express the Oct4, Sox2, Klf4, and c-Myc (OSKM) reprogramming factors in somatic cells, owing to their ability to randomly integrate in the host genome and potentially cause unpredictable mutations. To tackle this concern, new protocols ensuring higher safety of iPSCs have been established. Some of them eliminate the need for potentially transforming factors such as c-Myc or Klf4, while others rely on the use of nonviral vectors [68–75].

Numerous other issues also require attention. For instance, obtaining patient-specific iPSCs and differentiating both ESCs and iPSCs is expensive and time-consuming, and the methods to assess cell quality and purity need to be significantly improved and standardized.

Unquestionably, additional research is required to evaluate the benefits of treatment for human patients, and the long-term risk of tumor formation.

Sources of Stem Cells for Retinal Disease Treatment: The Use of Adult Stem Cells

Adult stem cells (ASCs) are characterized by a limited differentiation capability, giving rise only to a subset of cell types, belonging to a defined lineage. Adult stem cells from various specialized tissues (e.g., bone marrow, tooth pulp or adipose tissue) have been proposed as potential sources for the treatment of retinal diseases.

Among all of ASCs, retinal progenitor cells (RPCs) are located within the pigmented ciliary epithelium of adult mammals and are intrinsically efficient at differentiating into mature retinal cell types [76–78]. In vitro, they can generate PR precursors for cell replacement therapies, and some promising results have been published [79–81]. However, the migratory ability of RPCs is very poor, and obtaining them remains technically challenging. Additionally, they need to be harvested from early postnatal tissue, which raise considerable ethical concerns; consequently, their availability is extremely limited [82].

Given their ability to reprogram and differentiate into both RGCs and PR precursors, Muller Glia cells (MGCs) have been proposed as an alternative source of retinal stem cells. MGC-derived RGC precursors improve RGC function when transplanted in rodent models of NMDA-induced toxicity [83–87].

Bone marrow-derived cells (BMDCs) represent another important source of ASCs (Fig. 12.1). They are generally classified into two distinct subcategories: hematopoietic stem and progenitor cells (HSPCs) and mesenchymal stem cells (MSCs) [88]. HSPCs are multipotent cells able to differentiate into all blood cell types maintaining the hematopoietic tissue homeostasis, while MSCs differentiate into osteoblasts, chondrocytes, adipocytes and may serve as hematopoietic-supporting stromal cells [89, 90]. Moreover, it is becoming increasingly clearer that MSCs, under the appropriate environmental conditions, can generate cardiomyocytes, skeletal muscle cells, and even neural cells [91–93].

The protective effects that MSCs can exert on endogenous retinal cells are unquestionable [94, 95]. In fact, upon tissue injury or exposure to inflammatory microenvironments, MSCs can secrete a plethora of both anti-inflammatory cytokines and neurotrophic factors (NTFs), including NGF, BDNF, NT-3, NT4/5, CNTF, GDNF, and PDGF, that are critical for the repair of the injured tissue [96–100]. It is therefore not surprising that the vast majority of paracrine-mediated therapies are based on the use of MSCs. As already described in detail by Singer and Caplan [101], this "paracrine activity" is characterized by six main actions, namely: (1) anti-apoptotic signalling; (2) beneficial remodelling of the extracellular matrix; (3) activation and support of local stem cells; (4) increased angiogenesis to chronically ischemic tissue; (5) chemotaxis to promote leukocyte migration to the injured area; and (6) immunomodulation [101–103].

In particular, the immune-modulatory properties of MSCs are believed to be highly beneficial in the context of tissue injury and inflammation. In fact, MSCs can adopt a potent anti-inflammatory and protective action, being able to modulate the activity of both innate and adaptive immune cells [104–110].

In addition to being immunosuppressive, MSCs have negligible immunogenicity. In fact, they lack surface expression of the Major Histocompatibility Complex (MHC) class II and express low MHC class I levels [111–113]. Therefore, in addition to offering the possibility of autologous transplantation, MSCs are good candidates for the development of allogeneic therapies [94, 114].

In contrast to MSCs, ESC- and NSC-based therapies cannot be autologous by definition and some immunosuppression might be required for long-term allograft survival [64, 115–117].

Thanks to their high differentiation potential, BMDCs represent a very promising tool for therapeutic use in regenerative medicine. Evidence for the beneficial effects of HSPCs in the context of retinal disease is slowly but steadily accumulating. For instance, intravitreal injection has been proven beneficial in retinal dystrophy mouse models (Table 12.1) [118]. Additionally, allogeneic transplantation of HSPCs has been reported to ameliorate symptoms of severe neuromyelitis optica in at least two patients [119].

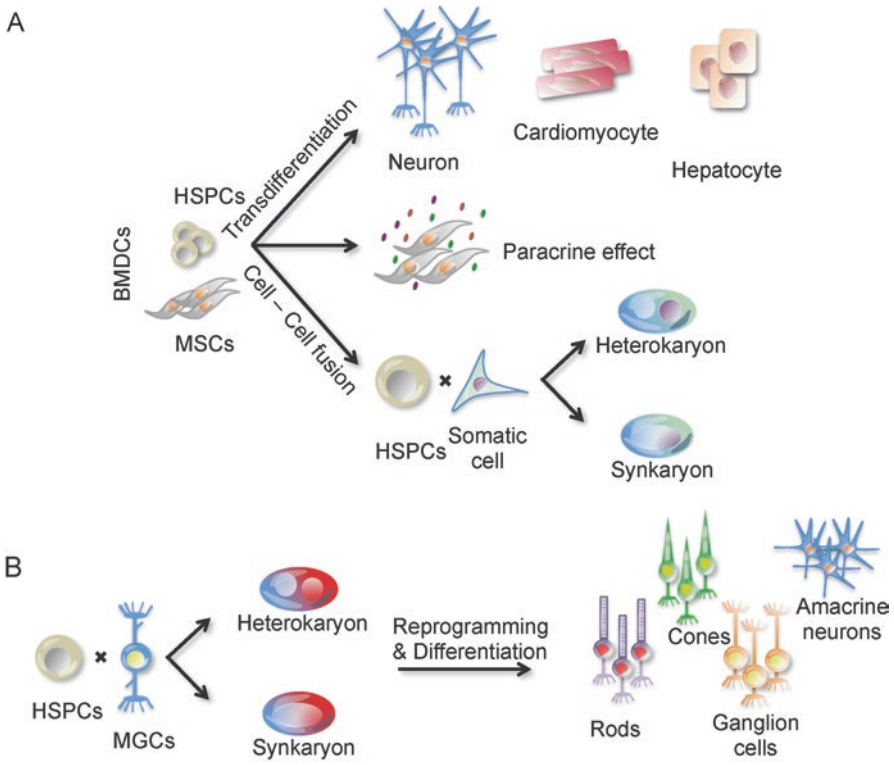

Fig. 12.2 Bone marrow-derived cells contribute to tissue regeneration. (**a**) Transplanted BMDCs can contribute to tissue regeneration via one of the three mechanisms: (1) transdifferentiation; (2) secretion of supportive paracrine factors; and (3) fusion with resident cells. (**b**) BMDCs predominantly fuse with Muller Glia cells (MGCs), generating hybrids (synkaryons and heterokaryons) that are transiently reprogrammed back to a precursor stage, proliferate, and rapidly commit to the neuroectodermal lineage, eventually differentiating into photoreceptors (rods and cones), ganglion cells, or amacrine neurons

In the late 1990s, some reports started introducing the idea that BMDCs could have a wider plasticity than previously assumed (Fig. 12.2a). This notion came from the observation that BMDCs could migrate to several organs and generate various cell types in response to niche-specific factors. For instance, BMDCs were reported to generate myocytes [120], cardiomyocytes [121, 122], hepatocytes [123, 124], neurons [125–127], and others [128]. Transdifferentiation (i.e., direct conversion of a specific cell to one of a different lineage) was originally believed to underlie the observed phenotype [129]. However, it was then proposed that cell fusion events between BMDCs and differentiated cells could also trigger these changes in cell identity (Fig. 12.2a).

Changing Cell Identity by Cell-to-Cell Fusion

Cell fusion events can occur between two cells with different functional and developmental stages and result in the combination of the two genomes into a mixed cytoplasm. Thus, the final phenotype is determined by a dosage combination of cytoplasmic and nuclear factors from each parental cell type.

Cell fusion can lead to the generation of either heterokaryons (i.e., cells with two distinct nuclei) or synkaryons (i.e., cells with one tetraploid nucleus, deriving from the two parental nuclei). Mammalian heterokaryons generate synkaryon daughter cells through mitotic division rather than fusion of the nuclear membranes [130, 131].

Both the synkaryon and the heterokaryon models have been used in vitro to study how two distinct genomes can affect one another. The two approaches are characterized by different limitations, but can often be complementary. For instance, synkaryon cells mix the two genomes, which makes it impossible to distinguish the contribution of each nucleus. In contrast, heterokaryons maintain the nuclei separated, allowing one to investigate how the two nuclear components can influence each other.

Pioneering studies using hybrid cells have demonstrated that gene expression is regulated not only by *cis*-acting DNA elements but also by *trans*-acting factors. Indeed, tumor suppressor genes [132, 133], cell cycle regulators [134], and somatic nuclear reprogramming [135] were initially studied in hybrid cells.

Overall, cell fusion-derived hybrids can provide important new insight into the mechanisms that mediate changes in cell identity and plasticity.

Somatic Cell Reprogramming by Fusion with Pluripotent Cells

Different stem cell types have been used to reprogram a fully differentiated cell back to a pluripotent state, including ESCs, embryonic germ cells (EGCs), and embryonic carcinoma cells (ECCs). In 1976, Miller and Ruddle reported that hybrid cells derived from fusion between pluripotent teratocarcinoma and thymus somatic cells could differentiate into the three germ layers. This first study indicated that pluripotency is at least partially maintained in these hybrid cells [135]. Indeed, the hybrids not only resembled ECCs morphologically but also reactivated the inactive X chromosome and expressed pluripotency markers such as alkaline phosphatase [136–139]. Although they were used for cell fusion studies and can form carcinoma that contain the three germ layers, ECCs have some limitations [140]. For instance, compared to ESCs, they retain low developmental capability, infrequently generate chimeras, and they are unable to contribute to the germline [141].

In 1981, it became possible to use ESCs in vitro [39, 40]. Since then, numerous studies have demonstrated the prominent reprogramming activity of these cells [142–146]. Furthermore, key pathways and their role during cell fusion-mediated reprogramming have been investigated. For instance, it has been shown that the

Wnt/ß-catenin and the AKT signalling cascade can both enhance cell fusion-mediated reprogramming in vitro [147–149]. Importantly, ESC-thymocyte-derived hybrids could contribute to all three germ layers in mouse chimeras at day E7.5 of development [144]. Despite their prominent reprogramming capability, ESCs cannot reprogram imprinted genes such as H19 and Igf2r.

In order to assess the effect of the two parental genomes during cell fusion-mediated reprogramming, heterokaryons between mESCs and human somatic cells were generated (B lymphocyte and fibroblast). Importantly, the mouse genome could reactivate human pluripotency factors by 24 h after fusion [150, 151]. Reprogramming was also shown to depend on activity of polycomb-group protein (PcG) and chromatin remodellers [152, 153]. These studies were further expanded to demonstrate that nuclear reprogramming of human somatic cells could be achieved via fusion with human ESCs [154].

EGCs established from primordial germ cells (PGCs) [155, 156] are characterized by a genome-wide hypomethylation on both imprinted and not imprinted loci [157, 158]. Although EGCs-derived chimeras show abnormalities, these cells maintain important pluripotent features, and they represent an attractive model to study DNA methylation in cell fusion-derived hybrids. EGCs can reactivate the silenced X chromosome and induce hypomethylation in the somatic nucleus. Importantly and in contrast to ESCs, EGCs have the unique capability of reprogramming even the parental imprinted genes H19 and Igf2r [159].

To conclude, ECCs, ESCs, and EGCs can all reactivate pluripotency genes and inactivate tissue-specific genes after cell fusion with somatic cells (even though in slightly different manners). The resulting hybrids can generate chimeras, although no evidence of germline contribution has been reported so far.

Nuclear Reprogramming After Fusion of Somatic Cells

The capability to induce changes in both gene expression and epigenetic markers after fusion is not a property unique to pluripotent cells. Indeed, differentiated cells also can change identity of the partner nucleus after fusion, pushing it into a different somatic state [160, 161]. Between the late 1960s and the early 1970s, it was established that fusion can induce changes in the specific gene expression pattern of a differentiated cell. For example, fusion-derived hybrids between mouse fibroblasts and hamster melanocytes or rat hepatocytes ceased to produce melanin and tyrosine amino-transferase, respectively [162, 163]. Experiments also showed that hybrids generated by fusion of albumin-producing rat hepatoma cells and either mouse fibroblasts or human leukocytes could synthesize albumin from both species [164, 165]. Similar studies showed that pigmented Syrian hamster cells hybridized with unpigmented mouse fibroblasts induced reactivation of the enzyme dihydroxyphenylalanine oxidase [166]. Later, mouse muscle cells were used to formally demonstrate that silent genes could be activated in mammalian heterokaryons. Mouse muscle cells were chosen because they are physiologically multinucleated

and they were hybridized with a number of human cells from all three germ layers. In particular, direct differentiation into muscle lineage was observed in heterokaryons derived from fusion between muscle cells and amniotic cells, keratinocytes (ectoderm), fibroblasts (mesoderm), or hepatocytes (endoderm) [160, 161, 167, 168]. The relative ratio of the lineage-specific factors contributed by the two cell types dictated the direction of the differentiation. In fact, a more recent study on hybrids derived from muscle cells and keratinocytes showed that an excess of primary keratinocyte factors can activate the keratinocyte program in muscle cells and vice versa [169].

These heterokaryon experiments have demonstrated that in differentiated cells, *trans*-acting repressors and activators from one partner can directly modulate gene expression in the other nucleus. In other words, differentiated cells possess the ability to change cell identity of the partner nucleus towards a different somatic state.

Cell Fusion and Tissue Regeneration

Cell fusion was first proposed as a mechanism for tissue regeneration in the late 1990s [170–172]. This was then confirmed in several mouse models. For instance, upon cell fusion, BMDCs could restore the metabolic function in a hepatic lethal mouse model with a mutation in the fumarylacetoacetate hydrolase gene [173–175]. The introduction of the Cre-LoxP system also facilitated the identification of fusion events in vivo. In fact, transplantation of Cre-expressing BMDCs into mice carrying a reporter gene that could be expressed only after excision of a LoxP-flanked STOP codon showed rare formation of hybrid cells with hepatocyte, cardiomyocyte, and Purkinje neurons [176–178].

The frequency of cell fusion is considerably increased in injured and inflamed tissues, highlighting the possibility that cell fusion might act as a physiological repairing mechanism in vivo [179]. Accordingly, several studies confirmed that BMDCs could participate to tissue regeneration via cell fusion with differentiated cells from the ecto-, meso-, and endoderm. Lineage tracing experiments show that myocytes [180, 181] and cardiomyocytes [182–184] can fuse in vivo with mature myeloid cells and BMDCs, respectively. Additionally, fusion-derived hybrids between BMDCs and vascular pericytes have been described. Stroke induction drastically enhanced cell fusion efficiency in the ischemic area [185]. Regarding the endoderm lineage, BMDCs were reported to form heterokaryons in vivo after fusion with lung pneumocytes [186] and intestine epithelial cells [187–189]. In addition to compensatory growth as a repair mechanism for the liver, BMDCs can fuse with hepatocytes and contribute to tissue regeneration [173, 175, 176, 190, 191]. Finally, in vivo hybrids characterized by regenerative potential were observed between BMDCs and different neuronal cell type such as Purkinje cells [177, 192–195] and dopaminergic neurons or glial cells [196].

Cell Fusion and Retinal Disease Regeneration

Over the past few years, evidence for the beneficial effects of cell fusion-derived hybrids in retinal disease models has been reported, suggesting that cell fusion could contribute to retina regeneration (Fig. 12.2b).

In particular, intravitreally transplanted HSPCs in an NMDA-damaged retinal model could fuse with retinal neurons and MGCs generating reprogrammed hybrids cells. Importantly, fusion of the transplanted cells was reported only in the context of tissue damage. Moreover, hybrids could efficiently survive and proliferate only if the transplanted HSPCs had been pretreated with an activator of the Wnt/b-catenin pathway. Indeed, this signalling pathway enhanced the reprogramming of the hybrids, which could then differentiate into ganglion and amacrine neurons, contributing to retina regeneration with some functional rescue [197]. Using a similar approach, Wnt-activated HSPCs were subretinally transplanted into either genetic (mice carrying a specific retinitis pigmentosa mutation) or drug-induced models of photoreceptor degeneration. In both models, MGCs not only were identified as the preferred fusion partner, but could also be reprogrammed in vivo. The newly generated hybrids proliferated and subsequently differentiated into photoreceptor cells, contributing to the rescue of some electrophysiological activity [198]. In both studies, HSPCs/MGC-derived hybrids were reported to promote retinal tissue repair via differentiation into mature ganglion, amacrine, and photoreceptor cells.

Cell-cell fusion between multipotent cells such as BMDCs and differentiated cells could contribute to the regeneration of different tissue types in vivo. One of the proposed mechanisms involves the spontaneous recruitment of endogenous BMDCs to the damaged tissue through the stromal cell-derived factor 1/C-X-C motif chemokine receptor type 4 (SDF-1/CXCR4) pathway. Indeed, the SDF-1/CXCR4 axis is required for BMDC mobilization from the niche to the injured tissue, and it has been associated with tissue repair in different contexts including liver, brain, and retina [191, 199–202].

In response to damage, BMDC mobilization could occur as a physiological response. This process would also open the possibility for new therapeutic strategies aimed at boosting endogenous repair, thereby overcoming problems associated with rejection of transplanted cells and their correct integration into the neural circuit.

Recent studies have advanced our knowledge of cell fusion-mediated regeneration. Many important questions remain to be answered, however, including: (1) Who is the more efficient fusion partner with fusogenic properties in the BMDC population? and (2) How do hybrid cells cope with high ploidy and differentiate into mature retinal neurons? The number of studies that broadly investigate these issues are quite limited. Nevertheless, there is some data suggesting that committed myelomonocytic cells are the most prominent fusion partners in liver model [175]. Furthermore, the same group proposed that ploidy reductions represent the mechanism that leads to the generation of genetically diverse daughter cells [203]. However, additional studies to evaluate whether these processes could be only liver-specific or more general are required.

Routes of Administration

Generally, three administration routes for stem cell therapy are available: intravitreal, subretinal, and systemic. For instance, MSCs are able to reach multiple organs following tail injection. Systemic delivery is limited, however, by cell entrapment in the lungs [204]. Additionally, the blood-retinal barrier (BRB) could prevent migration of MSCs into the host retina. Therefore, intravitreal or subretinal routes are typically preferred for the delivery of therapeutic stem cells in the context of retinal diseases.

On the one hand, being a minimally invasive procedure and allowing injection of a relatively large volume, intravitreal transplantation represents an attractive and popular choice. Indeed, a significant number of studies have shown that intravitreal injection of MSCs is beneficial in various models of glaucoma and retinal dystrophy [205]. Moreover, integration into the RGC layer and the INL appears to be favored following intravitreal transplantation vs. subretinal grafts [206].

On the other hand, subretinal implantation is a more complex and invasive procedure than intravitreal injection. Its success may depend on maintenance of the RPE and the outer blood-retinal barrier (BRB) integrity. If this integrity is compromised and the BRB is breached during surgery, the relative immune privilege of the subretinal space is lost, and immunosuppressive therapy is more likely to be necessary. However, the overall therapeutic effects of correctly performed subretinal transplantation appear to be greater and to last longer than those of intravitreal injections, especially in the context of PR degeneration [207]. Compared to the epiretinal approach, the subretinal microenvironment can better support and promote the differentiation of precursor cells towards PRs [57, 206]. Moreover, compared to intravitreal injections, subretinal grafts are generally associated with better migration and integration, also owing to the closer proximity of the transplant site to the injured retinal layers [207].

Overall, the suitability of each administration route may vary depending on the type and extent of tissue damage. Indeed, the disease type appears to profoundly affect the outcome of stem cell therapy in the eye. For instance, the success of rod PR transplants has been shown to vary across six different models of inherited PR degeneration and also with disease progression [208]. Encouragingly, transplanted cells seem to be able to survive and integrate even at late stage of degeneration, when the outer nuclear layer has been lost [208, 209].

It is important to stress that the majority of both animal studies and clinical trials have been performed using transplantation of cells in suspension. Recently, however, the use of biocompatible scaffolds has been introduced, holding the potential to boost the beneficial effects of the therapy [210–212]. For instance, a confluent RPE monolayer has been delivered using vitronectin-coated polyester membranes [210] and parylene C scaffolds [213]. This approach could be particularly important for the transplantation of RPE cells (e.g., in the case of AMD). In fact, cell suspensions may not properly attach and form a desired monolayer, clumping instead into rosettes [214].

In order to ameliorate engraftment efficiency, additional studies are required to combine the best cell type for transplantation with the appropriate biocompatible scaffold.

Migration and Integration for Cell Replacement

One of the major obstacles for the development of effective cell replacement therapies is illustrated by the low integration rate with the host retina. On average, only 0.03–0.2% of the transplanted cells are integrated into the host tissue [80, 215, 216] especially following intravitreal implantation [102, 205].

However, the integration efficiency varies depending on the transplanted cell type. For instance, transplanted NPCs are able to migrate extensively within the retinal layers. Their ability to differentiate into mature retinal cell types, however, is very poor [217, 218]. On the contrary, RPCs are extremely efficient at differentiating into retinal neurons, but their integration within the host retina remains very limited [44, 82, 219, 220]. It seems likely that the beneficial effects of transplantation could be much higher if a larger percentage of the transplanted cells were incorporated into the host retina.

The mammalian retina is characterized by the presence of natural physical barriers that may prevent the migration of transplanted cells. More specifically, the inner limiting membrane (ILM) is located below the ganglion cell layer (GCL) and is formed by the tight junction between endothelial cells and the foot processes of astrocytes and MGCs.

The outer limiting membrane (OLM), instead, consists of adherens junctions between MGCs and PRs. The ILM and OLM have been proposed to impede migration and integration of intravitreally [205] and subretinally transplanted cells, respectively. Consistently, transient pharmacological disruption of the OLM can increase the integration of transplanted photoreceptor precursors [221]. Interestingly, some diseases are associated with disruption of the retinal barriers, which could indirectly promote integration of transplanted cells [208].

The “impermeability” of the blood-retina barriers is degraded in the context of tissue injury and inflammation, when a process known as “reactive gliosis” occurs. Reactive gliosis is characterized by macrophage recruitment, microglia accumulation [222], and deposition of chondroitin sulfate proteoglycans (CSPGs) [223].

These events somehow contribute to the inhibition of transplanted cells migration and integration [224]. Therefore, inhibition of reactive gliosis could be critical for successful transplantation-based strategies for neuroprotection, replacement, and regeneration [225]. Indeed, migration and survival of transplanted cells can be promoted by adjuvant anti-inflammatory therapy and local degradation of the extracellular matrix with the enzyme chondroitinase ABC (ChABC), which selectively cleaves and inhibit CSPGs [87, 226]. In fact, CSPGs such as neurocan and glycoprotein CD44 have inhibitory properties with respect to both CNS regeneration (neurites and axonal growth) and migration/integration of transplanted cells [227, 228]. CSPGs inhibition can promote axonal regeneration [229, 230]. Consequently, it has been proposed that Chondroitinase ABC could represent a valuable tool to be incorporated in repair strategies and to be used in combination with different approaches. Indeed, ChABC can promote synaptogenesis between transplanted PRs and the host retina [231].

Conclusions and Open Questions

Retinopathies leading to vision incapacitation and blindness represent a substantial global burden and a growing problem that needs to be tackled.

Despite remarkable advances in the understanding of retinal disease pathophysiology, we currently lack effective treatments for this heterogeneous group of conditions. Various candidate strategies are being explored, ranging from gene therapy (especially for monogenetic hereditary diseases) to drug cocktails. Among them, stem cell therapy is advancing at a steady pace and stands out for the promising outcomes reported in both preclinical and early clinical studies.

Nonetheless, numerous questions in the field remain open. For instance, the optimal delivery routes for the various conditions have not been clearly established yet. Remarkably, the best option might depend on an unexpectedly high number of variables that include not only the type of injury/disease and cells being transplanted, but also the specific stage of the disease, the therapeutic aim (sight preservation or restoration), and the general conditions of the patient.

As discussed, the low rates of migration and integration can significantly dampen the beneficial effects of cell therapy. Our current knowledge of the signalling pathways and cues involved in migration and chemoattraction is quite limited. Therefore, a more comprehensive understanding of such mechanisms (ideally coupled with tailored manipulations of the retinal microenvironment) could lead to improved therapeutic outcomes and further development of stem cells as a curative tool.

Also, a growing number of studies now emphasize the beneficial effects of transplanting scaffolded cells, which will open up a new, exciting field that undoubtedly deserves deeper investigation.

Additional issues that still need to be satisfactorily addressed and clarified for clinical implementation of the therapy include: (1) the long-term risk of tumorigenesis and immunogenicity; (2) the establishment of ethically acceptable therapies; (3) the optimization and standardization of related protocols, with defined standards for the derivation of clinical-grade cells possessing a very well defined identity and lacking any kind of contamination from either microbial/infectious agents or tumorigenic cells. Also, some reports seem to suggest that the beneficial effects of stem cell therapy could only be temporary and disappear in the long run [232, 233].

Nonetheless, the outcome of clinical trials is slowly corroborating the promising results reported in animal models, indicating that stem cell therapy could realistically be feasible and highly beneficial for vision restoration in the context of retinal degeneration.

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Chapter 13
Clinical Trials of Retinal Cell Therapy

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Introduction

Decades of preclinical work in retinal regenerative therapy have now led to the initiation of clinical trials of stem cell therapy for retinal diseases. Stem cell therapy can be used to arrest or decelerate the degeneration of malfunctioning cells, or regenerate tissues following a genetic or acquired insult [1, 2]. There is increasing interest in using stem cell therapies for neurodegenerative diseases with the aim of replacing cells and tissues that are diseased. Exciting clinical progress in the translation of stem cell therapy from bench to bedside has occurred in such diverse organ systems as the liver, pancreas, spinal cord, and retina [3].

In the context of the retina, the functional consequence of cell loss is the partial or complete loss of vision. Stem cell therapy can be used to reduce the rate of visual decline over the lifetime of the recipient or to restore vision in areas of the visual field where blindness has set in [4]. In principle, these objectives can be achieved singly or in combination, depending on the severity and stage of disease in the recipient.

The retina constitutes an attractive target for stem cell-based therapies due to several reasons. It has direct accessibility, can be easily monitored using noninvasive techniques, has immune privileged status due to the blood-retina barrier, the contralateral eye can serve as an effective control in randomized studies, and advancements in retinal surgical

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techniques allow the transplantation of stem cells into specific locations in the eye [5–7]. This chapter will focus on providing an update on the status of registered clinical trials on stem cell therapy for retinal diseases.

Disease Targets

The degeneration of the light-sensitive layer of photoreceptor cells is the disease mechanism that underlies the leading causes of incurable blindness in developed countries, namely advanced nonexudative age-related macular degeneration (AMD) and inherited retinal degenerative diseases including retinitis pigmentosa and Stargardt disease. Currently, these conditions are the main disease targets of retinal stem cell treatment.

AMD, a leading cause of blindness in the developed world, exists in wet (exudative) and dry (nonexudative) forms. Safe and effective treatments for wet AMD have been developed, and the current mainstay of treatment is drugs that antagonize the action of vascular endothelial growth factor (VEGF) [8]. The dry form accounts for 80% of cases and, in advanced stages, is associated with retinal pigment epithelium (RPE) degeneration and geographic atrophy (GA) [9]. Although these diseases are associated with widespread death of cells in the outer retina including photoreceptors, RPE cells, and choroidal endothelial cells, the inner retina is mostly preserved [10–12]. Since the inner retina is mostly spared, restoring the outer retinal cells seems to be a promising approach to regain vision [13]. Although anti-VEGF therapy has transformed the landscape of AMD treatment, there is evidence that despite effective treatment of choroidal new vessels, these patients continue to lose vision due to progression of the dry form of the disease [14]. Thus, the advanced form of dry AMD (GA) can coexist with the advanced form of wet AMD [15]. Clearly, a treatment for GA represents an important unmet need in healthcare and is an attractive disease target for the development of regenerative therapy.

Retinitis pigmentosa is a group of heterogeneous diseases that feature progressive retinal degeneration, which often begins in childhood or teenage years. The first symptom typically is nyctalopia associated with progressive visual field loss from the periphery to the center, in many cases leading to total blindness. The rod photoreceptors are affected predominantly in the initial stages, followed by eventual involvement of the cone photoreceptors at which point high acuity central vision is lost [16].

Stargardt disease is the most common form of juvenile macular degeneration. It is caused by various mutations in the *ABCA4* gene that lead to the dysfunction and eventual death of photoreceptors and the retinal pigment epithelium (RPE) [17]. GA and Stargardt disease differ in important aspects, including genetic and environmental risk factors, inciting events, molecular cascades, and spatial distribution and tempo of cellular loss. Despite these differences, both conditions share the common feature of combined photoreceptor and RPE degeneration.

Rationale and Concepts Underlying Different Strategies for Clinical Application

The concept of retinal stem cell therapy is multifaceted, i.e., there are several different therapeutic paradigms that can be pursued. The treatment goal could be to preserve remaining vision in a patient affected by a mild condition or to restore vision in a completely blind patient. Depending on the goal selected, several options unfold. The first option is to define the cellular target for regeneration, followed by selecting the specific anatomical target zone for delivery, and then selecting the surgical delivery route that can achieve the delivery of the cellular substrate into the target zone in the safest and most effective way possible. A brief explanation of these variables is provided below to help the reader in developing an overview of strategies underlying the clinical trials in progress.

Treatment Objective: Preserving Versus Restoring Vision

The clinical spectrum of inherited and acquired retinal degenerative diseases is vast, and there can be marked differences in the severity of retinal degenerations between individuals. Certain mutations, for example, are known to cause visual dysfunction with the relative preservation of the cellular architecture of the retina for a long period. Others feature the early onset of cell loss, such that the tissue becomes atrophic at an early stage in the disease. Two patients with the same genetic mutation—even siblings in the same family—can show dramatic differences in the extent of retinal cell loss. The factors underlying these differences include genetic and environmental factors. Therefore, the patients could manifest relatively mild disease, i.e., with partial cellular degeneration in the retina or a more severe condition where almost all retinal photoreceptor and RPE cells have been lost in a localized region of the retina or across its entire topographical extent.

In the former case, a treatment strategy to preserve remaining vision will be useful. In this scenario, stem cells can be transplanted into the eye with the goal of neuroprotection. This mode of treatment relies on the fact that stem cell derivatives release diffusible chemical mediators that confer neuroprotective effects on partially compromised cells, thus delaying the time course of their decay. This paracrine or trophic approach could slow the time course of disease progression and delay the occurrence of severe visual loss. In the latter case of a patient with advanced retinal degeneration and severe vision loss, the paracrine strategy would not be applicable, because there are insufficient numbers of viable cells in the retina to protect. Instead, these patients would benefit from a treatment to replace or regenerate functional retinal photoreceptor cells.

Cellular Targets for Regeneration

The light-sensitive neurons of the outer retina are the photoreceptors. Both types of outer retinal photoreceptor cells, rods and cones—each serving different aspects of visual function—can degenerate. Typically, peripheral degeneration such as occurs in retinitis pigmentosa involves predominantly rod photoreceptor loss, whereas degenerations with a predilection for the central retina, such as AMD and Stargardt disease, involve significant cone loss. In cases of retinitis pigmentosa in which the mutation is known to occur only in rods (e.g., *P23H* mutation in rhodopsin), cones eventually die. The reasons for this outcome have not been established conclusively but may involve loss of trophic support from rods as well as altered glucose metabolism in cones secondary to rod cell death [18, 19].

Photoreceptor cells lie adjacent to the RPE layer, which commonly degenerates in these conditions. The cells comprising the vascular choroid also show degenerative changes in certain retinal degenerative diseases [20]. Therefore, potential targets for cellular regeneration for the treatment of retinal degenerative diseases include rod photoreceptor cells, cone photoreceptor cells, RPE cells, and choroidal vascular cells. The therapeutic ideal would be to replace all the damaged cellular components in the disease being treated.

Noncellular Target for Regeneration

The outer retina has noncellular structures that are thought to be critical for its physiological organization and function. Bruch membrane, a thin lamina composed of elastin and collagen, lies between the RPE and choroid [21]. Bruch membrane can show degenerative changes in retinal degenerative diseases [22, 23]. It has also been shown that aged Bruch membrane serves as a poor substrate for the attachment, survival, and optimal function of RPE cells in AMD [21, 24, 25]. However, Bruch membrane in younger patients with inherited retinal degenerations may not show the identical pathological alterations as those seen in AMD. Strategies to provide a synthetic replacement for Bruch membrane are included in certain clinical trials discussed below.

Target Zones for Transplantation and Associated Delivery Routes

The target zone for transplantation to the retina could be the intravitreal space or the subretinal space. The vitreous cavity normally contains the vitreous humor which might impede the free movement of transplanted cells although early clinical trials indicate that some cells can migrate through the vitreous gel and settle on the retina [26]. The vitreous gel can be removed partially or completely through a vitrectomy procedure.

Cells deposited into the vitreous cavity after a vitrectomy procedure will be able to move freely under the influence of gravity and settle on the retinal surface. The retinal surface is lined by the inner limiting membrane, which poses a barrier to the migration of cells from the vitreous cavity towards the photoreceptor layer [27, 28]. Therefore, cells introduced into the vitreous cavity are usually not intended to have their final location in the photoreceptor layer or outer retina. Instead, intravitreal cell delivery can be used to provide trophic support, as released chemical mediators can reach the target cells by diffusion.

The subretinal space is a potential space (i.e., not formed as a cavity) under normal physiological circumstances. However, the subretinal space is a natural plane of cleavage that can be intentionally separated by the introduction of fluid or by mechanical dissection. In the case of subretinal cell transplantation, the new photoreceptor and/or RPE cells can thus be positioned in their physiological location. As a result, subretinal cell delivery could be used for both rescue and replacement treatment strategies.

Tissue-Level Considerations

Cell therapy can be delivered as a bolus of dissociated cells suspended in aqueous medium or as a preformed sheet. The clinical trials described below use one of these two possible methods. A more detailed description of tissue-level considerations is available in the chapter in this book on surgical methods for transplantation.

Status of Clinical Trials

Pluripotent Stem Cells

Pluripotent stem cells (PSCs) are stem cells that can renew indefinitely and differentiate into any type of mature cell type of endodermal, ectodermal, or mesodermal origin. The two commonly used types of PSC include human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). Currently, there are several registered clinical trials on the ClinicalTrials.gov website that are studying the use of hESC derivatives as therapy for retinal degenerative diseases (Table 13.1).

In the United States, a pioneering study of the use of hESC in human retinal disease was initiated in 2011 by a company then known as Advanced Cell Technologies, later known as Ocata from 2014. In 2016, Astellas acquired Ocata and changed its name to Astellas Institute for Regenerative Medicine (AIRM). AIRM's cell product is hESC-derived RPE cells and is currently known as MA09-hRPE.

Table 13.1 Status of selected interventional clinical trials using pluripotent stem cells (data from ClinicalTrials.gov, first accessed January 2017, last accessed August 2018)

Study/identifier	Recruitment status	Country	Sponsor	Phase	Delivery route	Disease	Cell source	Cell target
Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (hESC-RPE) Cells in Patients With Stargardt's Macular Dystrophy (SMD) NCT01469832	Completed	United Kingdom	AIRM	Phase 1 Phase 2	Subretinal	SMD	hESC	RPE
Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients With Stargardt's Macular Dystrophy NCT01345006	Completed	United States	AIRM	Phase 1 Phase 2	Subretinal	SMD	hESC	RPE
Safety and Tolerability of Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients With Advanced Dry Age Related Macular Degeneration (Dry AMD) NCT01344993	Completed	United States	AIRM	Phase 1 Phase 2	Subretinal	Dry AMD	hESC	RPE
Safety and Efficacy Study of OpRegen for Treatment of Advanced Dry-Form Age-Related Macular Degeneration NCT02286089	Recruiting	Israel	Cell Cure Neurosciences Ltd.	Phase 1 Phase 2	Subretinal	Advanced dry AMD	hESC	RPE
Stem Cell Therapy for Outer Retinal Degenerations NCT02903576	Recruiting	Brazil	Federal University of São Paulo	Phase 1 Phase 2	Subretinal	AMD, Stargardt's disease, Exudative AMD	hESC	RPE
Clinical Study of Subretinal Transplantation of Human Embryo Stem Cell Derived Retinal Pigment Epitheliums in Treatment of Macular Degeneration Diseases NCT02749734	Active, not recruiting	China	Southwest Hospital, China	Phase 1	Subretinal	Macular degenerationSMD	hESC	RPE

Subretinal Transplantation of Retinal Pigment Epitheliums in Treatment of Age-related Macular Degeneration Diseases NCT02755428	Recruiting	China	Chinese Academy of Sciences	Phase 1	Subretinal	Nonexudative AMD	hESC	RPE
Study of Subretinal Implantation of Human Embryonic Stem Cell-Derived RPE Cells in Advanced Dry AMD NCT02590692	Active, not recruiting	United States	Regenerative Patch Technologies, LLC	Phase 1 Phase 2	Subretinal	Dry AMD, GA	hESC	RPE
Treatment of Dry Age Related Macular Degeneration Disease With Retinal Pigment Epithelium Derived From Human Embryonic Stem Cells NCT03046407	Recruiting	China	Chinese Academy of Sciences	Phase 1	Subretinal	Dry AMD	hESC	RPE
Examining the safety and feasibility of transplanting donor (allogeneic) iPSC-derived RPE Cells for wet AMD	Ongoing	Japan	RIKEN		Subretinal	Wet AMD	Allogeneic iPSC	RPE
Safety and feasibility of the transplantation of autologous induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) cell sheets in patients with exudative (wet-type) age-related macular degeneration	Halted	Japan	RIKEN	Phase 1	Subretinal	Wet AMD	Autologous iPSC	RPE
A Phase I/IIa, Open-Label, Single-Center, Prospective Study to Determine the Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (MA09-hRPE) Cells in Patients With Advanced Dry Age-related Macular Degeneration (AMD) NCT01674829	Unknown	Republic of Korea	CHABiotech CO., Ltd.	Phase 1 Phase 2	Subretinal	Dry AMD	hESC	RPE
Safety and Tolerability of MA09-hRPE Cells in Patients With Stargardt's Macular Dystrophy (SMD) NCT01625559	Unknown	Republic of Korea	CHABiotech CO., Ltd.	Phase 1	Subretinal	SMD	hESC	RPE

AIRM Astellas Institute of Regenerative Medicine, *AMD* age-related macular degeneration, *SMD* Stargardt macular dystrophy/degeneration, *GA* geographic atrophy, *hESC* human embryonic stem cells, *iPSC* induced pluripotent stem cells, *RPE* retinal pigment epithelium

Phase I/II open-label, non-randomized, dose escalation, multicenter trials using MA09-hRPE for Stargardt Disease were completed in the United States (NCT01345006) and the United Kingdom (NCT01469832). Additionally, a phase I/II trial was completed in the United States using hESC-RPE for atrophic AMD (NCT01344993). The launch of these pioneering clinical trials was a significant breakthrough in regenerative medicine.

The AIRM trials involved the delivery of MA09-hRPE, in the form of dissociated cells in suspension, into the subretinal space, following pars plana vitrectomy and transretinal cell injection. Patients were immune suppressed for 1 week prior and 12 weeks following the transplantation procedure. The immunosuppressive medications tacrolimus and mycophenolate mofetil were employed in this protocol. The primary end points were safety and tolerability. Nine patients were enrolled each for the Stargardt and AMD arms in the USA, and the results of these patients after median follow-up of 22 months were reported by Schwartz et al. in 2015 [29]. The phase I trial met its safety endpoint, as no adverse event resulted from the cell therapy, including adverse proliferation, rejection, or serious ocular or systemic safety issues. Moreover, all eyes except one either had improvement or no change in their best-corrected visual acuity [29]. These data were the first demonstration of the safety, survival, and possible therapeutic effect of PSC derivatives in patients with retinal degenerative diseases.

A 4-year follow-up of these 18 patients was subsequently published. There were no reported significant adverse events or safety issues related to the cells per se. There was one case of postoperative culture-positive (*S. epidermidis*) infectious endophthalmitis; however, the original hECS-RPE batch was negative for bacteria on Gram stain and culture. The patient received intravitreal antibiotic injection and antibiotic eyedrops, while the immunosuppressants were discontinued. The inflammation resolved over 2 months, and the vision returned to baseline by month 3. More than half of the patients showed a sustained, generally modest improvement in visual acuity and signs of possible cellular engraftment at the margins of areas of RPE-photoreceptor atrophy [30]. Mild (clinically unimportant) pigmented epiretinal membrane formation occurred in some patients.

Data have also been presented from the United Kingdom Stargardt disease arm of the trial (NCT01469832), in which 12 patients with Stargardt disease (STGD1) under the age of 53 years were enrolled. There was no clinical evidence of acute immune rejection, nor of uncontrolled proliferation locally or systemically. Borderline improvements in visual acuity were observed in four subjects; however, the improvements were either not sustained or were accompanied by similar changes in the fellow unoperated control eye. There were no changes in preferred retinal loci of fixation in any subject. Mean retinal sensitivity measured with mesopic microperimetry remained within the limits of variability in all comparison groups. The investigators detected reduced sensitivity and retinal thinning with high doses of RPE cell delivery. These data indicate the potential for harm especially when intervening in early disease stages or in areas of relatively well-preserved retina or RPE [31].

MA09-hRPE are not photoreceptor cells; hence the improvement in visual acuity in the US trial cannot be readily explained solely by the increased number of photoreceptor cells in the recipient retina after transplantation. However, there may be other mechanisms by which residual photoreceptor cell function in the recipient(s) was improved by the transplanted RPE. These mechanisms, including the regrowth of native photoreceptor outer segments and/or the development of new preferred retinal loci for fixation, may underlie the improvements in vision in the clinical trial subjects.

Currently, AIRM has ongoing noninterventional follow-up studies of their phase I/II trial for Stargardt disease in the United States (NCT02445612) and the United Kingdom (NCT02941991). AIRM also has a current ongoing follow-up study of their phase I/II trial for AMD in the United States (NCT02463344). These data will indicate if the favorable safety profile of these cells is maintained through 5 years, and relevant information could also emerge on whether the visual gains are sustained or continue to improve in the years following the transplantation procedure. AIRM has also registered a safety surveillance study (NCT03167203) to evaluate the occurrence of late onset (greater than 5 years post-treatment) safety events of special interest (adverse events which might have a potential causal relationship to hESC-RPE) in subjects who received hESC-RPE in an AIRM sponsored trial.

CHA Biotech in South Korea also started phase I trials using MA09-hRPE via subretinal transplantation for Stargardt disease (NCT01625559) and phase I/II trials for GA-associated AMD (NCT01674829). The current status of both these trials has not been updated in the last 2 years on the ClinicalTrials.gov website, and the completion date has passed. Cell Cure Neurosciences Ltd. in Israel is currently recruiting participants for their phase I/II trials to determine the safety and efficacy of hESC-derived RPE for advanced dry AMD (NCT02286089). Another phase I/II trial sponsored by the Federal University of Sao Paulo in Brazil is currently recruiting participants (NCT02903576). This trial is using hESC-RPE for AMD, Stargardt disease, and exudative AMD. Similarly, in China, a phase I trial by the Southwest Hospital (NCT02749734) and a trial sponsored by the Chinese Academy of Sciences (NCT02755428) are currently recruiting participants.

In Japan, RIKEN sponsored a study whereby autologous iPSC-derived RPE sheets were to be transplanted into the eyes of two patients with AMD. The first submacular transplant occurred in 2014 in a 77-year-old woman with no reported safety concerns. There was no evidence of serious complications over a 25-month follow-up period, and her vision remained stable [32]. However, mutations were detected in the iPSC-derived RPE cells that were produced for the second patient. Therefore, due to safety concerns, the sponsor decided not to proceed with the transplant in the second patient. According to the principal investigator of the trial and a published report, the main reason for the halt was certain regulatory changes introduced by the Japanese Government [32, 33]. It was announced at a press conference that a new clinical study will be started to establish a new treatment for exudative AMD using allogenic induced pluripotent cells transplanted as cell suspensions. This study will be led by the Kobe City Medical Center General Hospital in collaboration with the Osaka University, RIKEN, and the Kyoto University's Center for iPS Cell Research and Application (CiRA). The current plan

is described as transplanting 5 patients over a period of 2 years [34]. Recently, the first transplant of allogeneic iPSC-derived RPE cells was conducted in a man in his 60s with wet AMD. The iPSCs for the current study are obtained from CiRA's iPSC bank for regenerative medicine, which collects cells from healthy donors [35].

Pfizer, in collaboration with University College London, sponsored a phase I trial for acute exudative AMD (NCT01691261). Their trial used hESC-derived RPE in the form of a monolayer which contained RPE cells immobilized on a polyester membrane (PF-05206388). The first patient was treated at the Moorfields Eye Hospital in London in 2015. Two patients receiving these RPE sheet transplants have been reported [36]. Visual gains of 29 and 21 letters (over 12 months), respectively, occurred in the two patients, one of whom developed retinal detachment due to proliferative vitreoretinopathy, which may have been a complication of uncontrolled cell proliferation in the vitreous cavity. The detachment was repaired successfully. High resolution imaging indicated the establishment of functional interaction between the transplanted RPE cells and host photoreceptors as well as stable localization of the sheet transplant in the subretinal space following surgery. Only local immune suppression was used long term. There were no control patients (i.e., patients undergoing the surgical procedure for choroidal neovascularization absent the cell transplant) in this early phase study, which may be relevant [37].

Regenerative Patch Technologies is a company based in California that has developed technologies to produce and deliver a monolayer of stem cell-derived RPE cells on a scaffold. With funding from the California Institute of Regenerative Medicine (CIRM), the company initiated a phase I/IIA trial for patients with advanced dry AMD (NCT02590692). Their product for this trial is hESC-derived RPE attached to a small parylene membrane (CPCB-RPE1) delivered subretinally. The parylene membrane is nanoengineered to have diffusion properties similar to Bruch membrane. The density of the cells on the membrane reflects the approximate density of RPE cells in the human eye. The rationale behind the size of the membrane was to cover a substantial portion of the macula. Early clinical results in five patients are favorable [38]. The implant was placed successfully in four of the five subjects. Among patients receiving the implant, high resolution optical coherence tomography imaging suggested functional integration of the hESC-RPE and the host photoreceptors. One eye's vision improved by 17 letters and two eyes demonstrated improved fixation. Recently, Japan-based Santen Pharmaceutical has announced their decision to invest in Regenerative Patch Technologies for further development of their technology [39].

Bone Marrow-Derived Stem Cells

Bone marrow-derived stem cells (BMSC) do not directly replace retinal tissues. Instead, these cells produce cytokines that promote the survival of retinal cells and/or play a role in stabilizing the retinal vasculature [40]. Other mechanisms through which BMSC might preserve or restore retinal function include cellular differentiation,

paracrine effect, retinal pigment epithelium repair, and fostering differentiation of Muller cells into photoreceptors [41, 42]. Clinical trials of BMSC transplantation are listed in Table 13.2.

A preclinical study by Park et al. on a retinal disease model has described that GMP-grade CD34+ cells were present 4 months after intravitreal injection, and functional effects assayed with electroretinography were detectable until the eighth month after injection [43]. Based on these data, a phase I clinical trial was conducted to investigate intravitreally injected autologous CD34+ cells in six subjects with irreversible vision loss from a variety of retinal diseases, and this procedure was found to be safe and tolerable.

The University of Sao Paulo in Brazil completed phase I (NCT01068561) and phase II (NCT01560715) trials using autologous BMSC injected intravitreally for retinitis pigmentosa (RP). The preparation of autologous BMSC included aspiration of 10 mL of bone marrow from the posterior iliac crest under local anesthesia. This was followed by separation of mononuclear cells by Ficoll-Hypaque gradient centrifugation and suspension in buffered saline containing 5% human albumin. The final 0.1 mL that was used for the intravitreal injection contained a mean 1.68×10^4 autologous bone marrow-derived hematopoietic stem cells (CD34+) [44]. The phase I trial included 4 patients with RP and 2 patients with cone-rod dystrophy with poor vision (best-corrected visual acuity of 20/200 or less). There was no reported toxicity over a 10-month follow-up period [44]. Interestingly, quality of life data in 20 patients who received intravitreal autologous BMSC indicated that improvements in quality of life seen 3 months after the intravitreal injection were not sustained at 1 year [45]. The University of Sao Paulo also completed a phase I/II trial using BMSC delivered by an intravitreal injection for patients with macular degeneration (NCT01518127). Other phase I/II trials using BMSC in Spain, Jordan, India, Egypt, and Thailand, among others, are registered on ClinicalTrials.gov for which outcome data are not yet publicly available.

Another trial currently registered is the Stem Cell Ophthalmology Treatment Study (SCOTS). It is currently recruiting participants in the United States and the United Arab Emirates for its study using autologous BMSC for the treatment of retinal disease, AMD, hereditary retinal dystrophy, optic nerve disease, and glaucoma. This study is an open-label, non-randomized efficacy study with no placebo or control arm. It is self-described as the largest ophthalmology stem cell study registered with the NIH [46]. For the study, the bone marrow is aspirated from the posterior iliac crest and then separated to yield bone marrow cells within a concentrate [46]. In 2015, the investigators described a case of a 27-year-old woman with optic neuropathy who underwent this procedure and subsequently reported an improvement in her vision [47]. They also published another case report of a 54-year-old female with relapsing optic neuritis who had improvement in her vision after treatment [46]. Similarly, the Stem Cell Ophthalmology Study II (SCOTS 2) sponsored by MD Stem Cells is also recruiting participants in the United States and United Arab Emirates. These trials use autologous BMSC delivered via a combination of methods and for a number of different diseases. The SCOTS procedure is funded by the patients. The SCOTS trials were approved by an independent Institutional Review

Table 13.2 Status of selected interventional clinical trials using non-pluripotent cells (data from ClinicalTrials.gov, first accessed January 2017, last accessed August 2018)

Study/identifier	Recruitment status	Country	Sponsor	Phase	Delivery route	Disease	Type of cells
Autologous Bone Marrow-Derived Stem Cells Transplantation For Retinitis Pigmentosa (RETICELL) NCT01560715	Completed	Brazil	University of Sao Paulo	Phase 2	Intravitreal	RP	Autologous BMSC
Autologous Bone Marrow-Derived Stem Cells Transplantation For Retinitis Pigmentosa NCT01068561	Completed	Brazil	University of Sao Paulo	Phase 1	Intravitreal injection	RP	Autologous BMSC
Intravitreal Bone Marrow-Derived Stem Cells in Patients With Macular Degeneration (AMDCELL) NCT01518127	Completed	Brazil	University of Sao Paulo	Phase 1 Phase 2	Intravitreal	AMD Stargardt	BMSC
Clinical Trial of Intravitreal Injection of Autologous Bone Marrow Stem Cells in Patients With Retinitis Pigmentosa (TC/RP) NCT02280135	Completed	Spain	Red de Terraria Celular	Phase 1	Intravitreal	RP	Autologous BMSC
Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Age-Related Macular Degeneration (AMD) NCT01632527	Completed	United States	StemCells, Inc.	Phase 1 Phase 2	Subretinal	AMD GA	HuCNS-SC
Safety of a Single, Intravitreal Injection of Human Retinal Progenitor Cells (jCell) in Retinitis Pigmentosa NCT02320812	Completed	United States	jCyte, Inc.	Phase 1 Phase 2	Intravitreal	RP	Human retinal progenitor cells (jCell)

Phase 1/2a, Multicenter, Randomized, Dose Escalation, Fellow-Eye Controlled, Study Evaluating the Safety and Clinical Response of a Single, Subretinal Administration of Human Umbilical Tissue-Derived Cells (CNTO 2476) in Subjects With Visual Acuity Impairment Associated With Geographic Atrophy Secondary to Age-related Macular Degeneration NCT01226628	Completed	United States	Janssen Research & Development, LLC	Phase 1 Phase 2	Subretinal	AMD GA	Human umbilical tissue-derived cells (CNTO 2476)
Bone Marrow Derived Stem Cell Ophthalmology Treatment Study NCT01920867	Enrolling by invitation	United States, United Arab Emirates	MD Stem Cells		Retrobulbar, subtenon, intravenous, intravitreal, intraocular	Retinal disease, macular degeneration, hereditary retinal dystrophy, optic nerve disease, glaucoma	Autologous BMSC
Bone Marrow Derived Stem Cell Ophthalmology Treatment Study II NCT03011541	Recruiting	United States, United Arab Emirates	MD Stem Cells		Retrobulbar, subtenon, intravenous, intravitreal, intraocular	Retinal disease, AMD, retinitis pigmentosa, Stargardt disease, Optic neuropathy, nonarteritic ischemic optic neuropathy, optic atrophy, optic nerve disease, glaucoma, Leber hereditary optic neuropathy	BMSC
Safety and Tolerability of hRPC in Retinitis Pigmentosa (hRPCRP) NCT02464436	Recruiting	United States	ReNeuron Limited	Phase 1Phase 2	Subretinal	RP	Human retinal progenitor cells (hRPC)

(continued)

Table 13.2 (continued)

Study/identifier	Recruitment status	Country	Sponsor	Phase	Delivery route	Disease	Type of cells
A Prospective, Multicenter, Randomized, Study of the Safety and Efficacy of Intravitreal Injection of Human Retinal Progenitor Cells (jCell) in Adult Subjects With Retinitis Pigmentosa (RP) NCT03073733	Active, not recruiting	United States	jCyte, Inc.	Phase 2	Intravitreal	RP	Human retinal progenitor cells (jCell)
Clinical Trial of Autologous Intravitreal Bone-marrow CD34+ Stem Cells for Retinopathy NCT01736059	Enrolling by invitation	United States	University of California, Davis	Phase 1	Intravitreal	Nonexudative AMD, diabetic retinopathy, retina vein occlusion, retinitis pigmentosa, hereditary macular degeneration	Autologous CD34+ bone marrow stem cells
Early Phase I Study of the Safety and Preliminary Efficacy of Human Fetal Retinal Pigment Epithelial (fRPE) Cells Subretinal Transplantation in Age-Related Macular Degeneration (AMD) Patients NCT02868424	Active, not recruiting	China	The First Affiliated Hospital with Nanjing Medical University	Phase 1	Subretinal	Dry AMD	fRPE cells
Autologous Bone Marrow-Derived CD34+, CD133+, and CD271+ Stem Cell Transplantation for Retinitis Pigmentosa NCT02709876	Active, not recruiting	Jordan	Stem Cells Arabia	Phase 1 Phase 2	Intravitreal	RP	Bone marrow-derived CD34+, CD133+, CD271+ stem cells

An Open-label, Non-Randomized, Multi-Center Study to Assess the Safety and Effects of Autologous Adipose-Derived Stromal Cells Injected Intravitreal in Dry Macular Degeneration NCT02024269	Withdrawn	United States	Bioheart, Inc.		Intravitreal	Dry AMD	Autologous adipose-derived stromal cells
Feasibility and Safety of Adult Human Bone Marrow-derived Mesenchymal Stem Cells by Intravitreal Injection in Patients With Retinitis Pigmentosa NCT01531348	Enrolling by invitation	Thailand	Mahidol University	Phase 1	Intravitreal	RP	Bone marrow-derived mesenchymal stem cells
Clinical Study to Evaluate Safety and Efficacy of BMMNC in Retinitis Pigmentosa NCT01914913	Unknown	India	Chaitanya Hospital, Pune	Phase 1 Phase 2	Unclear	RP	Autologous bone marrow-derived mononuclear stem cell
Safety Study of Use of Autologous Bone Marrow Derived Stem Cell in Treatment of Age Related Macular Degeneration NCT02016508	Unknown	Egypt	Al-Azhar University	Phase 1 Phase 2	Intravitreal	AMD GA	Autologous BMSC

AMD age-related macular degeneration, *GA* geographic atrophy, *BMSC* bone marrow-derived stem cells, *HuCNS-SC* human central nervous system stem cells, *RP*: Retinitis pigmentosa, *fRPE* fetal retinal pigment epithelium

Board (IRB) that was not associated with an academic medical center. A recent article, published in Ars Technica raised concerns about stem cell treatment trials that are patient-funded, mentioned the SCOTS and SCOTS 2 studies [48].

Retinal Progenitor Cells

The transplantation of human retinal progenitor cells (hRPC) for RP comprises a promising avenue for slowing disease progression and possibly also restoring vision function. hRPC are obtained from fetal eyes between 16 and 18 weeks of gestation [49, 50]. Following expansion, they can differentiate into retinal cells before or after transplantation. Differentiated hRPCs have been shown to express photoreceptor markers including opsins [51–53]. Low oxygen conditions have shown to further extend their expansion phase [53]. Since these cells can be expanded several times, they form an attractive modality for generating a large number of donor cells for use in clinical application [54].

jCyte announced the launch of a phase 2b trial (NCT03073733) to study the safety and efficacy of its jCell treatment for RP after receiving a matching grant of $8.3 million from CIRM. jCell contains hRPC that are intended to rescue and possibly replace diseased photoreceptor cells. The trial will deliver the treatment through a single intravitreal injection and the visual function of participants will subsequently be monitored as the primary goal [55]. This trial is being conducted in collaboration with the University of California Irvine and CIRM. jCyte has also completed its phase 1/2a trial (NCT02320812) using jCell for RP.

A phase I/II trial funded by ReNeuron, which also uses hRPC for RP, is currently recruiting participants in the United States (NCT02464436). The mode of delivery for this trial is through subretinal transplantation, which differentiates this effort from the intervention proposed by jCyte. This study is based at the Massachusetts Eye and Ear Infirmary in Boston. Recently, FDA approved ReNeuron's cryopreserved hRPC therapeutic candidate [56].

Fetal Retinal Pigment Epithelial Cells

The first fetal retinal transplant into the anterior chamber of rat eyes was reported in 1959 [57]. Transplantation of allogenic fetal RPE (fRPE) grafts in humans was reported later on; however, without immunosuppression these grafts lead to leakage on fluorescein angiography and eventual fibrosis [58]. The rejection rates were found to be lower in dry AMD as compared to wet AMD [59]. An early phase 1 trial (NCT02868424) involving subretinal administration of fRPE cells for dry AMD is currently ongoing in China. All subjects in this trial will receive oral immunosuppressive agents after transplantation with the dosage and timing strictly regulated based on the condition of immune rejection.

Human Umbilical Tissue-Derived Cells

In an experimental study by Lund et al., human umbilical tissue-derived cells (hUTC) demonstrated preservation of both photoreceptors and vision in a mouse model of retinal degeneration. These cells also showed a population doubling capacity without incurring karyotypic changes [60]. In a subsequent study by Cao et al., hUTC were found to repair RPE phagocytic dysfunction in retinal degenerations through various cellular mechanisms involving bridge molecules and receptor tyrosine kinase ligands [61]. Janssen Research and Development LLC has completed a phase 1/2a trial (NCT01226628) involving subretinal administration of human umbilical tissue-derived cells (CNTO-2476) for GA secondary to AMD. In this study, a high rate of complications related to the delivery procedure was reported: six retinal detachments and 13 retinal perforations, likely resulting in off-target delivery in these cases. Where the cells were contained in the subretinal space, CNTO-2476 appeared to be well tolerated with potential visual function improvements [62].

Human Central Nervous System Stem Cells

Human central nervous stem cells (HuCNS-SC) possess the potential to be used as a therapy for various central nervous system disorders. These cells have demonstrated their multipotent ability in vitro by differentiating into various cells of the central nervous system [63]. StemCells, Inc. is a company that utilized a technique involving monoclonal antibody-based high-speed cell sorting to purify and bank HuCNS-SC [64]. Phase I and II trials sponsored by StemCells, Inc. have been completed (NCT01632527). These trials used HuCNS-SC transplanted directly into the subretinal space for AMD. However, StemCells, Inc. has terminated its study of HuCNS-SC for geographic atrophy of AMD due to a business decision [65].

Autologous Adipose-Derived Stem Cells

Zuk et al. reported adipose tissue as a source of multipotent adult stem cells as an alternative to bone marrow-derived stem cells [66]. Adipose-derived stem cells are much easier to obtain as compared to mesenchymal stem cells from the bone marrow. A clinical trial sponsored by Bioheart Inc. was listed on the clinical trials website in 2013 with the aim of assessing the safety and effects of autologous adipose tissue-derived stem cells given via intravitreal injection for dry AMD (NCT02024269). This trial was withdrawn in 2015 before beginning enrollment due to the report of serious adverse effects in three women with AMD who received bilateral intravitreal injections of stem cells in a stem cell clinic and developed

profound visual loss. Although these women were not enrolled in any trial, the stem cell clinic where the procedures were performed was scheduled to be the study site for the withdrawn trial [67]. The experience of these patients attracted the attention of regulating authorities and the medical world regarding the devastating effects of unregulated and self-funded stem cell treatments [48].

Conclusion

The FDA has not yet approved any retinal stem cell therapy product for clinical use. However, great progress has been achieved in this area of biomedicine, as several stem cell studies have progressed from the preclinical phase to phase I and II clinical trials. The clinical trials aim to either protect patients from further vision loss through a neuroprotective treatment paradigm or, in contrast, to restore vision in blind patients in a replacement treatment paradigm. A wide variety of cell sources are being investigated, including pluripotent stem cells (hESC and iPSC) and non-pluripotent cell types such as bone marrow cells, umbilical cells, and fetal retinal progenitor cells. While favorable safety outcomes have been reported from large and well-run clinical trials, concerns have arisen from reports of serious adverse events from others, underscoring the need for thorough and effective institutional oversight of any experimental application of stem cell therapy prior to FDA approval. The next decade may well bring news of favorable efficacy signals from these clinical trials and eventual FDA approval of safe and effective retinal stem cell treatments to preserve or restore vision in people with retinal degenerative diseases.

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Chapter 14
Future Directions

Mandeep S. Singh and Marco A. Zarbin

During the last five years, retinal cell therapy research has transitioned from the preclinical arena to early stage clinical trials. Thus far, data from these clinical trials [1–6] indicate that it is feasible to deliver cell therapy products into the vitreous cavity or subretinal space in human eyes. Importantly, there have been no significant safety concerns related to the transplanted cells or synthetic basement membrane substrates where the latter have been used as part of a composite patch. Uniformly, transplanted cells have not shown clinically important uncontrolled proliferation nor tumor formation in the eye or at extraocular sites. There have also been no reported occurrences of immune rejection or severe intraocular inflammation.

Several adverse events have arisen, however, from the vitreoretinal surgery procedures used for cell delivery, particularly for subretinal delivery [3, 6]. Complications have included retinal perforation, retinal detachment, and proliferative vitreoretinopathy. Further development of retinal cell therapy as a safe treatment will necessitate refinement of surgical protocols in order to avoid such surgical complications.

The Janssen trial of human umbilical tissue cell (hUTC) transplantation provides an example of an iterative improvement in surgical technique [6]. The target area for surgical delivery in this trial was the subretinal space, and the investigators used a flexible microcatheter, the iTrack 275, to deliver the cells. In the first few patients, the investigators encountered a high rate of surgical complications, so they introduced a surgical improvement consisting of retinal endoscopy in order to more completely visualize the surgical entry site into the intraocular compartment.

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Upon introducing this technique, retinal perforations were detected (and, presumably, treated) more frequently, thus avoiding an even higher postoperative retinal detachment rate in subsequent patients [2].

The complexity of the cell therapy substrate is also likely to evolve. Thus far, each clinical trial has featured the transplantation of only one cell type—for example, umbilical tissue cells [6], bone marrow cells [2], or embryonic stem cell-derived retinal pigment epithelium cells [3–5]. The use of a single cell type for therapy may suffice when the goal is for the cells to release trophic factors that exert a protective effect to slow down the rate of degeneration in the diseased recipient. If the goal is to regenerate totally or partially atrophic areas in the retina, however, such as in geographic atrophy, then therapeutic objective should be to remedy the multiple cellular and noncellular deficiencies that exist in such patients [7].

Currently targeted cellular deficiencies are mainly those of photoreceptor cells and retinal pigment epithelial cells. Noncellular deficiencies include pathologic changes in Bruch membrane [8, 9]. Thus, the ideal therapeutic construct would include at least two cell types—photoreceptor cells and retinal pigment epithelium cells—placed on a synthetic membrane that would act as a replacement for the native Bruch membrane. Such a composite multicellular cell patch would more closely approximate the natural multilayered anatomy of the human macula.

Data from the clinical trial in Japan [1], wherein induced pluripotent stem cells were used to generate retinal pigment epithelium cells for transplantation, indicate that ex vivo cell culture protocols may give rise to potentially deleterious mutations within the therapeutic cell product. This observation calls for the need to develop robust safety and quality assays to detect genetic changes in the cells intended for therapeutic transplantation. This issue is of particular concern with the use of induced pluripotent stem cell technology, where the source material may show significant variability between patients.

Achieving optimal immunosuppression is another aspect of cell-based therapy that is likely to improve. Whereas the earliest clinical trial(s) [4] utilized systemic immunosuppression with multiple agents administered perioperatively, a more recent trial [3] has attempted long-term local immunosuppression via an intraocular steroid implant. The advantage of this approach, if successful, is that it avoids the systemic morbidity associated with systemic immunosuppression. This morbidity is likely to be a significant issue for elderly patients with age-related macular degeneration receiving cell therapy. Minimization of systemic immune suppression and use of local immune suppression seem likely to improve the safety of retinal cell therapy without compromising efficacy.

Regarding the progress of clinical trials, patients, doctors, and industry partners eagerly anticipate inception of phase 3 clinical trials that will assess the efficacy of retinal cell therapy. Completion of these trials in a timely manner requires appropriate clinical endpoints to judge outcomes. Best-corrected visual acuity has been the gold standard clinical trial endpoint for almost all major ophthalmic clinical trials involving retinal disease; however, this measure may not be an appropriate endpoint for retinal degenerative diseases such as retinitis pigmentosa and Stargardt disease [10]. Visual acuity typically changes very slowly in retinitis pigmentosa with 20/20

vision preserved until late in the disease. Thus, best-corrected visual acuity may not show a detectable change with therapy within a realistic timeframe [11]. Furthermore, in patients with very low baseline vision, visual acuity may not represent a clinically meaningful measure of success.

Other functional endpoints could be considered, such as visual field area, retinal sensitivity as assayed by microperimetry, or visual-guided mobility [12] and luminance detection [13]. Regarding anatomical endpoints, possible measures include changes in the ellipsoid zone area [14–16] or a different proxy measure of photoreceptor health on the cellular level using high-resolution optical coherence tomography imaging. The advent of adaptive optics [17, 18], which enables one to image individual photoreceptors in vivo, may provide a novel clinical trial endpoint based on photoreceptor cell density and/or alignment [19], although such a measure has yet to be standardized in patients with advanced retinal degenerative disease. Technical difficulties with fixation also may pose a challenge for deployment of this technology in this clinical setting. As novel imaging technologies become refined, e.g., quantum dots that permit assessment of the metabolic state of the cell, additional noninvasive measures of transplant function in situ will be possible [20, 21].

Even at this early stage of cell-based therapy development, it is evident that greater regulatory oversight is needed to minimize the damaging consequences of unregulated direct-to-consumer interventions [22, 23]. Such interventions have resulted in dire consequences including severe bilateral visual loss in some patients. Perhaps paradoxically, greater progress in properly conducted clinical trials may exacerbate this need. As national regulatory authorities increase surveillance of centers offering these treatments [24–26], it will also be important to provide clinicians and patients with more information on the current status and scientific foundations of retinal cell therapy clinical trials.

Retinal cell-based therapy has the potential to restore relatively high degrees of visual acuity to blind patients. It also may be sight-preserving for patients at a relatively early stage of disease progression. In fact, cell-based therapy has restored useful vision in laboratory animals with retinal degenerative disease. We are now engaged in the transitional phase of refining these technologies for clinical application in human patients with similar or identical diseases. Thus, the promise of safe and effective cell-based treatment for retinal degenerative diseases is finally taking shape. The next decade is likely to be marked by continued progress in this therapeutic endeavor with the first positive results from pivotal human trials.

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