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Cell Stem Cell
Review

Regenerating Eye Tissues to Preserve and Restore Vision

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Ocular regenerative therapies are on track to revolutionize treatment of numerous blinding disorders, including corneal disease, cataract, glaucoma, retinitis pigmentosa, and age-related macular degeneration. A variety of transplantable products, delivered as cell suspensions or as preformed 3D structures combining cells and natural or artificial substrates, are in the pipeline. Here we review the status of clinical and preclinical studies for stem cell-based repair, covering key eye tissues from front to back, from cornea to retina, and including bioengineering approaches that advance cell product manufacturing. While recognizing the challenges, we look forward to a deep portfolio of sight-restoring, stem cell-based medicine.

> "In general, when a naturalist has a little disposition to reflect, he is risen soon by thought above the objects that his eyes behold…. Who knows if this will not lead to some discovery that will perfect medicine and surgery?"—Charles Bonnet (Bonnet, 1779)

Introduction

It is apt to begin with a quote from Charles Bonnet, a pioneering researcher in regenerative studies who would lose his vision, curtailing a most promising career. Bonnet performed some of the earliest work on regenerating animals, those capable of "recovering from cutting," in the 1740s. Bonnet saw the possibilities that regeneration presented for science and medicine and was particularly enthralled by the idea of a "germe" within tissues that was capable of regeneration—perhaps a harbinger of the stem cell concept. An accomplished naturalist by the age of 25, he preferred nature and philosophy to his profession as a lawyer, but his sight began failing, altering the course of his studies. He documented the life changes due to vision loss, including the unnerving visual hallucinations that he attributed to changes in visual perception, which are now known as Charles Bonnet syndrome. The profound physical and emotional challenges of vision loss—compounded by loss of independence and compromised life quality, career prospects, and earning opportunities—are strong motivators to identify new ways to restore sight, and remain so today.

The human eye is a remarkable structure produced from the coordinated development of multiple tissues, with contributions of neuroectodermal, ectodermal, and mesodermal origin (Graw, 2010). Compromising the function of any of these major ocular tissues can lead to blindness (Figure 1). There is a notable history of trail-blazing work in ocular medicine, exemplified by tissue transplantation, the use of laser therapy, and the recent approval of the first gene therapy for RPE-65-based retinal dystrophy. We may consider that the eye is also pioneering cell therapy for advanced disease involving cell loss, and the possibility for combinations of cell and gene therapy in the future is exciting. However, before we can achieve these therapeutic ends, we must address significant hurdles in cell manufacture, surgical delivery, and functional restoration. Here we describe the clinical status of ocular regenerative therapies, review some of the most promising preclinical work, consider the challenges, and look to the future, to what might be accomplished in the next decade. Our approach is to review the state of the field from cornea to retina, summarizing the advances for each target tissue, as illustrated in Figure 2.

Cornea: Repair of Three Layers

The cornea is the window to the visual system, acting as a barrier and a lens to focus light entering the eye. Loss of corneal integrity and transparency, resulting in reduced vision, afflicts more than 23 million individuals worldwide (Flaxman et al., 2017). To date, stem cell-based applications have demonstrated restoration of function in each of the three major corneal layers: the superficial epithelium, the stroma, and the inner endothelium.

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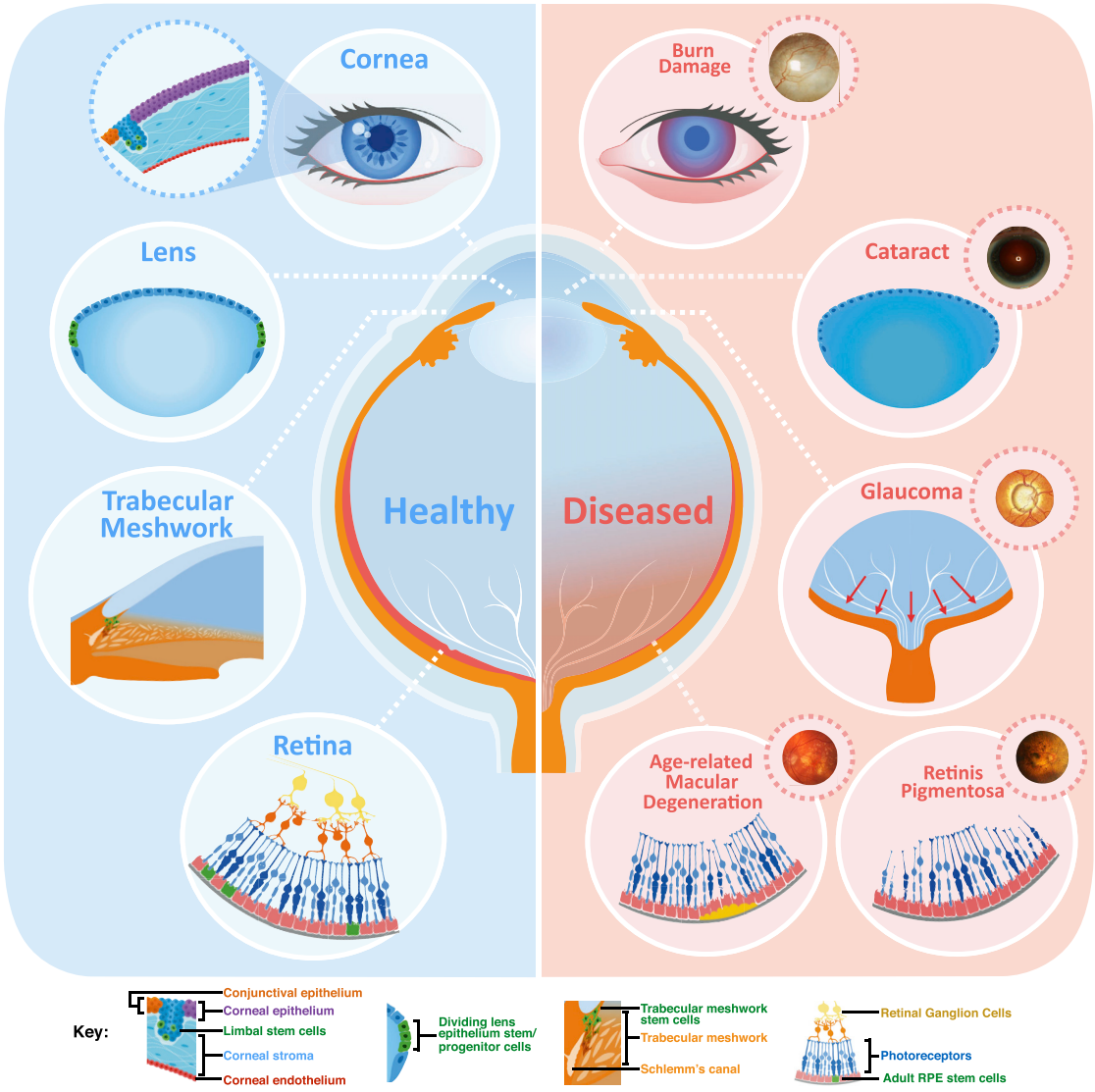

Figure 1. Ocular Stem Cells in Health and Disease
Schematic showing the location of corneal limbal, lens, trabecular meshwork (TM), and RPE stem cells, and depicting ocular disorders that are being targeted by regenerative therapies.

The corneal epithelium (CE) is a self-renewing tissue that is maintained by stem cells localized at the peripheral limbus (Figure 1). Limbal stem cells produce transient amplifying daughter cells that regenerate the epithelium through centripetal migration. Destruction of the limbal stem cell niche results in limbal stem cell deficiency (LSCD). Congenital or acquired LSCD impairs CE renewal, resulting in progressive opacification, chronic ulceration, conjunctivalization, and neovascularization with accompanying pain, blindness, and disfigurement. Rescue of LSCD by transplantation of stem cell-containing limbal biopsy tissue was demonstrated as early as 1965 (Barraquer, 1965). A modified technique of transplanting limbal tissue fragments adhered to an amniotic membrane has more recently exhibited a high rate of success in 190 patients who maintained the previous good transparency in the underlying stroma (Sangwan and Sharp, 2017).

Pellegrini and colleagues pioneered clinical trials using cultured autologous limbal epithelial cells for transplantation (Pellegrini et al., 1997). After more than 10 years of clinical follow-up, remarkably, corneal regeneration could be achieved in over 70% of cases if the limbal cell cultures contained a sufficient number (over 3%) of limbal stem cells, which were detected as holoclones expressing high levels of the p63 transcription factor (Rama et al., 2010). The limbal stem cells were obtained by enzymatic extraction from a small biopsy and cultured on a fibrin glue support. Recent regulatory approval of this technique in the EU represents the first available application of stem cells for ocular therapy. Cultured autologous limbal epithelial cell transplantation has been reproduced by many groups, using varied supports, culture conditions, cell sources, and carriers, the most common now being amniotic membranes containing growth factors (Tsai et al., 2000; Zhang et al., 2016).

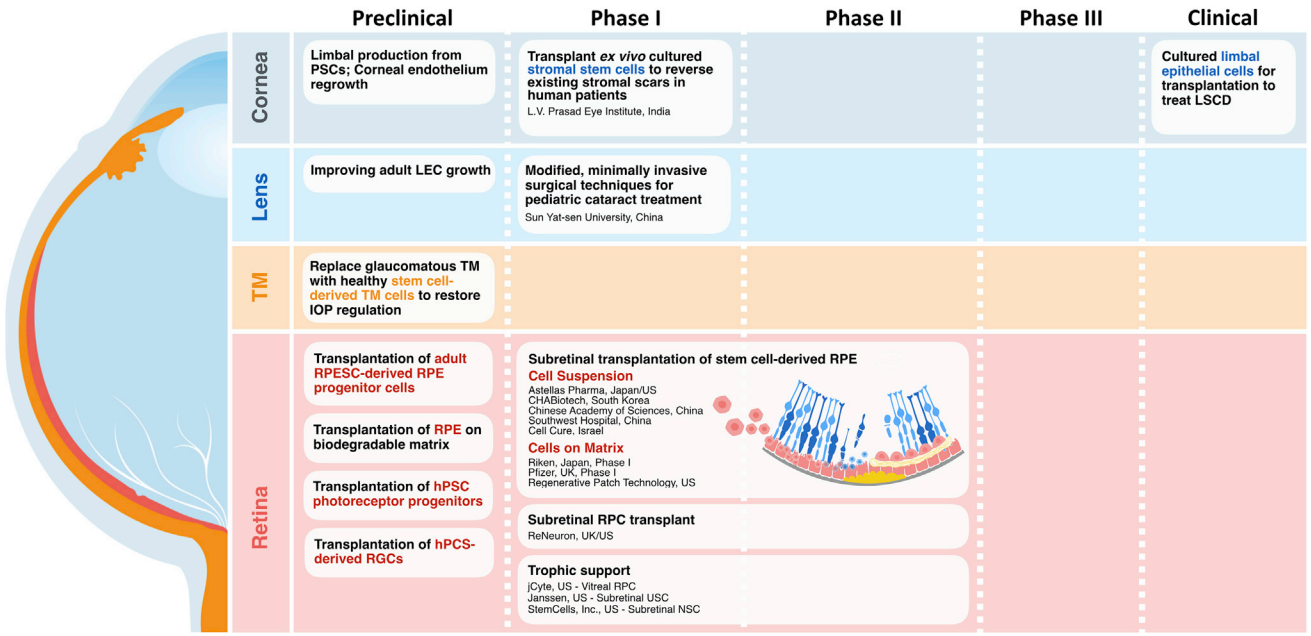

Figure 2. Clinical Pipeline for Cell-Based Therapies for Eye Disease

Corneal limbal stem cells and stromal cells have advanced to clinical practice, while corneal endothelial stem/progenitor cells are in preclinical stages of development, as are trabecular meshwork stem cells. Phase I and II clinical trials are ongoing for RPE replacement using PSC-RPE in suspension or on a matrix, while adult RPESCs are in late-stage preclinical development. Subretinal neural stem cell (NSC), umbilical cord stem cell (UCSC), and intravitreal or subretinal RPC clinical trials aim to provide trophic support to prevent retinal cell death.

Limbal stem cells provide a self-organizing system that faithfully and stably reproduces corneal epithelial physiology over time. In cases in which autologous cells are limiting, allogeneic cultivated limbal epithelial transplantation has been proposed as a therapeutic option, although the infiltration of host inflammatory cells often occurs in the absence of recipient stem cells, which can result in an inflammatory response and failure (Cheng et al., 2017; Shortt et al., 2010, 2014). The use of cell surface markers such as ABCB5 to purify limbal stem cell cultures is currently under development to potentially improve efficacy (Gonzalez et al., 2018; Ksander et al., 2014), although the role of ABCB5 in human epithelial regeneration remains to be fully investigated. Progress to direct pluripotent stem cell (PSC) differentiation to limbal epithelial lineages (Hanson et al., 2017; Mikhailova et al., 2016) promises to overcome current bottlenecks limiting limbal stem cell availability, to manufacture product for a wider population of LSCD patients.

The bulk of the cornea consists of the stroma, a connective tissue with a unique transparent extracellular matrix (ECM). The stroma is populated by quiescent, mesenchymal cells, termed keratocytes, which maintain stromal transparency in healthy tissue. In response to trauma or infection, however, stromal cells produce long-lasting opaque scar tissue (Funderburgh et al., 2001, 2003). The incidence of corneal blindness due to stromal scarring greatly exceeds that of LSCD (Oliva et al., 2012; Ontario and Health Quality Ontario, 2008; Shortt et al., 2010), and only 7% of affected individuals worldwide have access to corneal transplantation, underscoring the great unmet medical need. Adult mesenchymal cells derived from stroma (sMSCs) produce a collagenous matrix *in vitro* that contains the molecular components and achieves the organization typical of the native stromal tissue (Du et al., 2007; Wu et al., 2014). sMSCs are localized in the limbal stroma, where they are closely associated with epithelial stem cells (Chen et al., 2011; Dziasko et al., 2014; Higa et al., 2013; Massie et al., 2015). *In vitro*, co-culture with human sMSCs improves the expansion and clonogenicity of human limbal epithelial stem cells (Xie et al., 2012), suggesting sMSCs may be a component of the limbal niche complex that supports self-renewal. Epithelial and stromal stem cells are phenotypically and functionally distinct. Restoring cells to address LSCD does not fully reverse existing stromal opacity, nor is a healthy corneal stroma sufficient to revert the pathologic epithelial phenotype in the absence of limbal epithelium.

Transplantation of tissue generated *in vitro* from human sMSCs into corneal pockets in animal models yields a transparent stroma (Syed-Picard et al., 2016), which highlights the potential utility of corneal sMSCs to produce tissue for surgical replacement. sMSC transplantation can also remediate stromal scarring. Injection of human sMSCs into the cornea of lumican knockout mice, which have hazy stroma due to deficient collagen fibril organization, results in remodeling (Du et al., 2009). Even more striking, sMSCs completely prevent stromal scarring in a mouse corneal debridement wound model (Basu et al., 2014). The mechanism of scar prevention in mice was found to depend on the ability of sMSCs to block infiltration of neutrophils into the cornea by secretion of the protein TSG-6 (Hertsenberg et al., 2017). These data suggest that the anti-scarring effects of sMSCs are related to their immunomodulatory properties. Using a similar approach, an ongoing clinical trial (NCT02948023) examines the ability of sMSCs to reverse existing stromal scars in human patients.

The corneal endothelium is a post-mitotic epithelial monolayer lining the innermost cornea that maintains stromal hydration required for transparency. Endothelial decompensation is responsible for most (around 75%–80%) of corneal opacity in the United States. Cells with clonogenic potential demonstrated in adult corneal endothelium suggest that a dormant population of replication-competent endothelial progenitor cells is present (Amano et al., 2006; Yokoo et al., 2005). Experimental approaches to initiate endothelial regrowth include freeze damage to the central region in combination with application of the ROCK inhibitor Y-27632, which may activate dormant endothelial progenitor cells (Koizumi et al., 2014). Furthermore, transplantation of corneal endothelium has been demonstrated in human trials (Kinoshita et al., 2018). Derivation of functional corneal endothelial layers has also been reported from sMSCs (Hatou et al., 2013; Inagaki et al., 2017) and from PSCs (McCabe et al., 2015; Song et al., 2016; Zhao and Afshari, 2016), and transcriptome profiling suggests high fidelity to primary corneal endothelial cells (McCabe et al., 2015), raising the possibility of a plentiful supply of autologous cells for personalized restoration of corneal endothelium.

Due to the simplicity of its structure, accessibility, and avascularity, the cornea serves as an ideal tissue to advance stem cell-based regenerative medicine applications. This has yielded the first approved ocular stem cell therapy for limbal restoration. The results of ongoing clinical trials will guide future stem cell therapies to restore vision to the millions of individuals suffering from corneal disorders that currently lack effective treatment options.

Lens Regeneration Using Endogenous Stem Cells

Along with the cornea, the transparent, biconvex lens in the anterior segment of the eye refracts light, constantly adjusting shape or ''accommodating'' to focus (Figure 1). The lens surface is covered entirely by the lens capsule, a protective, supportive basement membrane. Immediately under the capsule on the anteriorlateral surface is the lens epithelial cell layer. Developing lens fibers arise from the equatorial cells of the lens epithelium (Figure 1), which over time form thin, elongated, anuclear cells full of cytoplasmic crystallin proteins (Cvekl and Zhang, 2017).

Lens regeneration can occur in lower vertebrates during developmental stages, while adult regeneration is limited to some urodele amphibians, through transdifferentiation of corneal or iris tissues (Barbosa-Sabanero et al., 2012; Tsonis and Del Rio-Tsonis, 2004). In mammals, including rabbits, cats, and dogs, removal of lens contents while leaving the capsule can result in proliferation of residual lens epithelial stem/progenitor cells (LECs), thus generating new lens fibers (Tsonis and Del Rio-Tsonis, 2004; Gwon, 2006). In humans, however, regrowth of lens tissue after cataract removal is disorganized and does not yield regeneration of a functional lens.

Cataract is a leading cause of blindness in the world (Stevens et al., 2013) and is treated by surgical lens extraction with implantation of an artificial intraocular lens (IOL). After cataract surgery, however, visual axis opacification due to disorganized regeneration of the remaining LECs often leads to impaired vision, which in most cases can be corrected by a laser procedure. Notably, pediatric patients experience robust LEC proliferation on the IOL surface. Therefore, current pediatric cataract surgery is performed to maximally remove LECs that may regrow and compromise transparency of the visual axis. A large, 6 mm diameter central capsulorhexis is opened in the center of the anterior lens capsule, and then most of the lens material is removed, including the PAX6+, SOX2+ LECs (Cvekl and Zhang, 2017) associated with the lens capsule, thereby also limiting the possibility of functional lens regeneration.

To promote useful lens regeneration, a minimally invasive surgical method has been developed using a small, eccentric capsulorhexis to reduce wound size and move the opening to the lens periphery, thus preserving LEC stem cells and the integrity of the lens capsule (Lin et al., 2016). This new technique demonstrated functional lens regeneration in rabbit and macaque models. A clinical trial enrolling infants with bilateral congenital cataract showed that the small capsule opening healed within 1 month and a transparent lens structure began to regenerate within 3 months. From follow-up to 8 months, the lens attained normal central thickness with accommodative power. While the regenerated lenses had imperfections, the visual axis was mostly clear with corresponding visual acuity improvement. This groundbreaking pediatric cataract surgery is still in early stages of evaluation and requires additional study to address possible complications such as cataract reoccurrence due to underlying genetic defects and the risk of developing amblyopia, impaired vision due to inappropriate visual stimulation during the months required for lens regeneration (Liu et al., 2018; Solebo et al., 2018; Vavvas et al., 2018).

Could we expand this regenerative approach to adult cataract surgery affecting tens of millions of people? A key challenge is that age-related declines in the LEC's regenerative capacity may necessitate augmentation, for example by incorporating biomaterials. Furthermore, current adult cataract surgery is safe and rapidly restores functional vision, whereas lens regeneration, if at all possible, could require several months in adults. Nevertheless, accelerated lens regeneration has the potential to restore both lens clarity and accommodative capacity to address both presbyopia and cataract.

Trabecular Meshwork Restoration: The Major Pathological Site of Primary Open Angle Glaucoma

Primary open angle glaucoma represents about 90% of all the glaucomas, affecting about 1% of the total population. It is defined by an open, normal appearing anterior chamber angle (Figure 1) with raised intraocular pressure (IOP). The condition is painless, and by the time vision loss is detected, many retinal ganglion cells (RGCs), the projection neurons that carry signals from retina to brain, are irreversibly lost. IOP is determined by the continuous generation of aqueous humor by the ciliary processes (inflow) and its elimination (outflow); the trabecular meshwork (TM), together with Schlemm's canal, collector channels, and aqueous veins, constitute the major outflow pathways in the eye. The TM is located in the anterior chamber angle between the cornea and the iris (Figure 1) and is crucial to provide flow resistance to the aqueous humor and thus control IOP. The current treatment of glaucoma is focused on lowering IOP by pharmacological reduction of aqueous humor production or by laser-based and surgical procedures to provide an aqueous humor outflow bypass (Fan and Wiggs, 2010). An alternative or complementary strategy is to target the TM, and

pharmacological agents that act on the TM to increase outflow e.g., ROCK inhibitors, adenosine agonists, and statins, are being developed (Kopczynski and Epstein, 2014) and some have shown encouraging results in the clinic (Hoy, 2018). Understanding the mechanisms leading to TM cell dysfunction and loss in the glaucomatous eye can illuminate novel strategies to target this primary pathological site (Wang et al., 2001).

The TM has three distinct regions: the inner uveal meshwork, the deeper corneoscleral meshwork, and the deepest, outermost juxtacanalicular connective tissue (JCT). The JCT region of the TM and/or the adjacent endothelial lining of the inner wall of Schlemm's canal generate the major flow resistance (Johnson, 2006; Tamm, 2009). Flow through the TM structure is segmental, i.e., non-uniform, and divided into high- and low-flow regions. Importantly, the cellularity of the TM is altered with age and in glaucoma, with accumulation of ECM, altered junctions between cells of the TM and Schlemm's canal, and TM cell death, together associated with IOP elevation. Aged and glaucomatous TM cells show signs of senescence and are susceptible to reactive oxygen species (ROS)-induced damage (Chhunchha et al., 2017; Saccà et al., 2007).

Stem cell therapy has the potential to restore TM function, as well as protect the optic nerve from further damage (Chamling et al., 2016). Replacing glaucomatous TM cells with healthy stem cell-derived cells may restore the microenvironment and make it suitable for reparative proliferation and functional regulation of aqueous outflow. TM stem cells were discovered at the region where the TM inserts (Figure 1) and have been isolated by side-population cell sorting, clonal culture, and sphere culture (Gonzalez et al., 2006; Kelley et al., 2009; Yun et al., 2016). They are multipotent and differentiate into TM cells *in vitro* and *in vivo*, where they home to the TM region and can regenerate the TM structure and reduce IOP in mouse models (Du et al., 2012, 2013, 2016). In addition, induced PSC (iPSC)-derived TM cells were shown to restore IOP-related homeostatic function in a human anterior segment *ex vivo* model (Abu-Hassan et al., 2015) and in glaucoma mouse models *in vivo* (Zhu et al., 2016, 2017). Moving forward, preclinical studies focused on stem cell-derived TM cell transplantation are needed to establish their safety profile and potential activity to move to clinical trial in glaucoma patients. Additionally, stem cell fabricated TM models provide opportunities for better understanding trabecular outflow physiology, glaucoma pathophysiology, and drug testing.

Differentiation and Transplantation of Retinal Ganglion Cells

Optic neuropathies represent a cluster of diseases in which RGC axons are injured in the optic nerve. As with other damage or degeneration in the central nervous system (CNS) of adult mammals including humans, RGCs fail to regenerate their axons and the cell bodies die at some point thereafter. Given that there are no endogenous stem cells that normally regenerate retinal neurons in adults, the loss of RGCs can lead to permanent vision loss, irrespective of the cause of their death, such as traumatic damage, ischemia, optic neuritis, glaucoma, or other diseases. Glaucomatous optic neuropathy is a leading cause of irreversible blindness in the world and vision restoration remains a major unmet need.

Stem and progenitor cells offer opportunities to treat glaucoma and other optic neuropathies. One approach is to transplant cells that secrete trophic factors, to preserve existing cells and prevent further cell loss via a neuroprotective strategy. For example, fetal human retinal progenitor cells (RPCs) implanted into the vitreous are being channeled through preclinical testing and regulatory preparation in anticipation of neuroprotection clinical trials (Klassen, 2016), and these may prove valuable in treating glaucoma. MSCs transplanted into the vitreous body in a rodent model of glaucoma showed considerable neuroprotection against RGC death without integration of the transplanted stem cells (Johnson et al., 2010). Bone marrow-derived stem cells have been tested in humans in a Phase I safety trial for retinal diseases (not optic neuropathies) with no overt inflammatory or other adverse event (Park et al., 2014).

Cell replacement therapy faces a much tougher set of hurdles related to appropriate RGC differentiation and integration, and the field is just beginning to make significant preclinical progress in these areas. To restore visual function, stem cells would have to properly differentiate into RGCs, integrate with presynaptic amacrine and bipolar cells in the retina to receive visual information, grow their axons down the damaged or diseased optic nerve, and properly connect with appropriate targets in the lateral geniculate nucleus and other regions of the brain (Moore and Goldberg, 2010).

How can we promote RGC differentiation from stem cells? Recent progress has been made in identifying the key transcription factor regulators relevant to RGC differentiation during normal development, and this benefits production of RGCs from human stem cells. In the absence of *Math5* expression, RGCs fail to differentiate from retinal progenitors during development (Brown et al., 2001), and mutations in *ATOH7* (*Math5*) and *Six6* can lead to optic nerve hypoplasia (Prasov et al., 2012; Schmitt et al., 2009). In the absence of *Brn3* expression, RGCs die shortly after differentiation (Badea et al., 2009). *Sox4* and *Sox11* were found to be critical for RGC differentiation from rodent RPCs as well as human iPSCs (Al-Shamekh and Goldberg, 2014; Chang et al., 2017). Other approaches to RGC differentiation including chemically defined media and signaling pathway manipulation have also demonstrated success in generating RGCs and even RGC subtypes (Daniszewski et al., 2018; Gill et al., 2016; Ohlemacher et al., 2016; Teotia et al., 2017). Use of embryonic stem cell (ESC) lines engineered to express reporter genes under control of RGC-specific promoters has also facilitated differentiation studies (Sluch et al., 2015, 2017). Progress has also occurred in promoting RGC differentiation from retinal Muller glia, which may provide a local, endogenous source of cells for replacement therapy (Wan and Goldman, 2016; Wilken and Reh, 2016). Important questions remain whichever cell source is used, including whether the variety of RGCs will arise naturally after transplantation *in vivo*, or if it will be necessary to direct the specification of different RGC subtypes.

Recent progress in preclinical models of RGC transplantation demonstrated successful local integration, synapse formation, responsiveness to light, and growth of a small number of axons down the optic nerve to the brain (Venugopalan et al., 2016). Data suggesting that these experiments were not confounded by protein transfer included visualization of growth cones at increasing distances with survival time and differences from

host RGCs in dendrite morphology and response to light, but this issue will need ongoing attention in future work. Extending this work to preclinical models of glaucoma is essential. It is not yet clear if the injured or degenerating environment will prove toxic to incoming cells or if the prior loss of endogenous RGCs provides favorable conditions for engraftment by clearing niches for new cells to enter. In addition to local integration within the retina, it is also critical to promote RGC axon regeneration through the optic nerve toward the brain, another research area with much recent progress including discovery of transcription factors and signaling pathways that strongly promote axon growth (Benowitz et al., 2017; Moore et al., 2011; Trakhtenberg et al., 2018). Outcomes of these studies will help determine whether the new RGCs and/or the host environment will need to be treated to overcome the existing barriers to optic nerve axon growth and brain reconnection.

The move to clinical trials can proceed after appropriate preclinical proof-of-concept and safety studies. Neuroprotection and visual function enhancement trials in glaucoma and other optic neuropathies are going forward for drug candidates such as neurotrophic factors (Chang and Goldberg, 2012), which could also be used to augment a cell-based treatment. These are facilitated by novel surrogate biomarkers that may provide a faster indication of biological effect and potential for clinical efficacy (Scoles et al., 2014), which should likewise facilitate rapid translation to safe human testing.

Photoreceptor Transplantation and Cytoplasmic Material Transfer

Photoreceptor cells are the light-responsive cells of the retina and consist of rods, which are activated in low light, and cones, which are activated in bright light of specific wavelengths. Cones are concentrated in the macula, the central region of the retina that provides high-acuity color vision. Photoreceptors are terminally differentiated neurons, and once lost, they are not replaced. In the developed world, conditions such as advanced retinitis pigmentosa (RP), age-related macular degeneration (AMD), and diabetic retinopathy—all characterized by photoreceptor loss—are the main causes of registered blindness. Photoreceptor replacement therapy is under development to provide a therapeutic approach for patients who lack effective treatment options. The requirement to establish synaptic connectivity is a significant challenge, but transplanted photoreceptors only need to make a few short synaptic connections to bipolar cells in order to connect to the remaining circuitry. Further, the macula occupies a small area and relatively few functional photoreceptor cells may be required for useful vision (there are, for instance, only 20,000 cones in the foveola, the cone-rich zone at the center of the macula), so even low-efficiency photoreceptor transplantation may result in clinical benefit.

A fundamental requirement for the development of effective photoreceptor transplantation is the establishment of robust protocols that permit the generation of large numbers of bona fide photoreceptors from renewable cell sources. Building on the groundbreaking work by Yoshiki Sasai, who showed that ESCs cultured in 3D suspension in the presence of Matrigel spontaneously form self-organizing retinal organoids (Eiraku et al., 2011; Nakano et al., 2012), a number of laboratories have developed culture protocols for generating large numbers of postnatal-stage PSC-derived photoreceptors that can be used for transplantation (Assawachananont et al., 2014; Decembrini et al., 2014; Gonzalez-Cordero et al., 2013; Homma et al., 2013; Kruczek et al., 2017; Meyer et al., 2011; Reichman et al., 2014; Zhong et al., 2014). Over the last decade, numerous preclinical studies have transplanted predominantly rod photoreceptor precursors derived from stem cells into mouse models (for reviews, see Aghaizu et al., 2017 and Santos-Ferreira et al., 2017). The transplanted stem cell-derived photoreceptor progeny are capable of restoring aspects of visual function. Until recently, it was understood that the observed improvements in visual function, at least in models in which some endogenous photoreceptors remained, were due to donor cells integrating in the host retina. Over the last few years, however, we have come to realize that the apparent integration of donor cells was instead due to exchange of RNA and protein between donor and host cells (Pearson et al., 2016; Santos-Ferreira et al., 2016; Singh et al., 2016). Although the mechanisms for cytoplasmic material transfer (CMT) are still unknown, the acquisition of proteins may render host cells functional. Observed rescue of visual function following transplantation of human ESC (hESC)-derived retinal cells (Lamba et al., 2009) was associated with GFP-labeled cells that bore striking resemblance to host photoreceptors and are likely to be the result of material transfer. While CMT may account for much of the reported outcomes of photoreceptor transplants, a recent study demonstrated that the transplantation of cone photoreceptors results in both CMT and donor cell integration, and the relative contribution of these two processes depends upon the host retinal environment (Waldron et al., 2018); for example, where there are no remaining photoreceptors, CMT is impossible.

Several new studies involving transplantation of mixed populations of dissociated hPSC-derived retinal cells and hPSC-derived sheets in models of end-stage retinal degeneration have shown improvements in visual function (Mandai et al., 2017a; Singh et al., 2013). hPSC-derived photoreceptor sheets have also been transplanted in non-human primate retinae (Shirai et al., 2016). Although it is more difficult to generate and isolate large numbers of PSC-derived cones for transplantation, this is an important objective because humans rely mostly on cones for high-acuity vision. A recent study involving the transplantation of hESC-derived cones in an end-stage model of RP resulted in the formation of nascent outer segment-like structures and basal processes that extended toward endogenous interneurons (Gonzalez-Cordero et al., 2017). While it is not yet possible to conclude whether these cones are light responsive or if the basal terminals represent synaptic connections, the transplantation of human PSC-derived cones represents a major step toward clinical application. Further optimization and demonstration of robust functional connectivity along with the development of good manufacturing practice (GMP)-compliant methods to quantify the purity, viability, and developmental stage of hPSC-derived photoreceptors are needed to enable the initiation of clinical trials. Such methods may include the use of cluster of differentiation (CD) cell surface markers to enrich rod (Lakowski et al., 2018) and particularly cone precursor cells (Welby et al., 2017), circumventing the use of reporter markers in transgenic hPSC lines that cannot be used clinically. More accurate staging of

hPSC-derived photoreceptors might also be achieved by comparing transcriptomes with those recently reported for developing human retina (Hoshino et al., 2017; Welby et al., 2017). While some groups have already started to develop GMP-compliant differentiation protocols that generate stage-matched, homogeneous populations of hPSC-derived photoreceptors for clinical testing (Reichman et al., 2017; Sridhar et al., 2013), further developments are needed. These include the development of GMP processes to manufacture sheets of photoreceptor cells, methods for enriching cultures for cone photoreceptors, and the development of bioreactor technologies with improved aeration and distribution of nutrients (DiStefano et al., 2018) and enclosed automated systems to enable GMP-compliant scale up of cell manufacture. Notwithstanding the need for further development, we anticipate that the first clinical trials of stem cell-derived photoreceptor transplantation to treat advanced retinal degeneration will be initiated within the next 10 years. Future developments might include, for instance, the use of gene-modified cells that have been engineered to repair genetic disease (Burnight et al., 2017) or have reduced immunogenicity or enhanced connectivity.

Photoreceptor Replacement and Rescue by Human Fetal Retinal Progenitor Cells

In addition to cell replacement strategies to treat diseases in which photoreceptors die, the neuroprotective approach is also being pursued: to transplant cells that release trophic factors to preserve rather than replace the function of surviving host photoreceptors. StemCells Inc., an early leader in the field of regenerative medicine, was the first to use human fetal neural stem cells to treat retinal disease. A 2012 Phase 1/2a study recruited 15 patients with dry AMD. This first-in-human study aimed to preserve vision by rescuing host photoreceptors through a paracrine mechanism involving the secretion of cytokines. Progenitor cells (CD133+) expanded from early fetal brain tissue were injected into the subretinal space (Cuenca et al., 2013; McGill et al., 2012) with oral immunosuppression, and this early study demonstrated safety. Other groups are pursuing stem or progenitor cells derived from the retina itself, with the hypothesis that such a cell has a higher chance of rescuing or replacing diseased photoreceptors.

Two groups (ReNeuron and jCyte) have taken up this approach using human RPCs, proliferating cells derived from fetal retina, characterized largely by the expression of key surface markers. Human RPC transplantation has two potential mechanisms of action: photoreceptor rescue and photoreceptor replacement, which target different injection sites—the vitreous cavity and the subretinal space, respectively. jCyte has initiated a clinical trial based on neuroprotection by injecting RPCs into the vitreous of patients with RP. The mechanism of action is based on the hypothesis that one or more cytokines secreted by RPCs may preserve photoreceptor function. The Phase 1/2a trial was completed and a Phase 2b trial consisting of 70 participants is currently underway. ReNeuron is transplanting a somewhat different cell type, also termed an RPC, into the subretinal space. These RPCs are cultured under specific conditions (Aftab et al., 2009; Baranov et al., 2013; Kundu et al., 2018; Lawley et al., 2015), including low oxygen (Baranov et al., 2014), in order to preserve their ability to produce photoreceptors, and they are then injected under the retina (Bharti et al., 2014) with the goal of replacing lost rods and cones (Huang et al., 2014) in patients with RP or cone/rod dystrophy. This work has also progressed through a Phase 1 study, with a larger Phase 2a study underway. In both jCyte and ReNeuron clinical trials, no systemic immunosuppression is used.

As with all transplants of stem or progenitor cells, there remains a risk of uncontrolled proliferation following transplant. However, progenitor cells derived from adult or fetal tissues are inherently safer than those derived from ESCs or iPSCs, due to lack of contaminating pluripotent cells and associated decreased risk of tumorogenesis. Another lesson we have learned from these first-in-human studies is that stem and progenitor cells of early CNS origin appear to be safe and well tolerated, with no evidence of rejection, inflammation, or proliferation. With Phase 2 studies underway, we will learn whether RPCs have the capacity to preserve or improve vision in patients with retinal degeneration. Important next steps include isolation and transplantation of cone-specified RPCs, as well as application of tissue engineering principles such as the use of polymer scaffolds or co-grafts of RPCs and retinal pigment epithelia for macula repair.

RPE Replacement and Repair

The major clinical targets of retinal pigment epithelial (RPE) replacement therapy are AMD and Stargardt's disease (SD), macular disorders that involve early loss of the RPE cells that support overlying photoreceptor cells and the neural retina. The human macula is a small region of the central retina, around 5 mm in diameter. Damage to this area is debilitating due to impairment of high-acuity vision. Dry AMD is a highly prevalent condition in which gradual loss of RPE cells is followed by photoreceptor cell loss and diminished vision. About 10% of dry AMD cases progress to wet AMD, with rapid vision loss due to ingrowth of choroidal vessels that are normally separated from the retina by Bruch's membrane. Although treatment is available for wet AMD, there is no effective treatment for earlier stage dry AMD (Zarbin et al., 2014). SD is a less prevalent autosomal recessive juvenile macular degeneration that lacks effective treatment. SD is most frequently associated with an *ABCA4* mutation that reduces ATP-binding transporter processing of all-trans retinal during the visual cycle in photoreceptor cell discs. This defective pigment processing leads to the accumulation of toxic lipofuscin and A2E lipids, which cause RPE and photoreceptor cell atrophy (Molday, 2015). Clinically, both AMD and SD are characterized by early pigment mottling and later hypotrophy of the macula RPE with accompanying loss of central vision. Stem cell-derived RPEs are being developed to replace the RPE cells that are lost in each condition. After cell transplantation, vision measures and retina imaging provide valuable outcome measures to evaluate stem cell-based RPE replacement therapies.

Considerable progress has been made toward efficient protocols that direct differentiation of both ESCs and iPSCs to RPE progeny. The potential for unlimited expansion makes PSCs an attractive source cell. Subretinal transplantation of PSC-derived RPEs (PSC-RPEs) rescues vision in animal models of degenerating retina (Carr et al., 2009; LaVail, 2001; Lund et al.,

2006; McGill et al., 2017; Riera et al., 2016; Sun et al., 2015; Wu et al., 2016). These encouraging preclinical findings led to clinical trials of ESC- and iPSC-RPE transplantation in the United States, Japan, China, Israel, the United Kingdom, and South Korea (Figure 2). Reports from early phase trials transplanting PSC-RPE suspensions (Schwartz et al., 2016; Song et al., 2015) are encouraging, with an ESC-RPE cell suspension found safe for AMD (NCT01344993) and SD (NCT01469832). Transplantation of ESC-RPEs resulted in long-term macula pigmentation, possibly due to transplanted cell persistence or stimulation of endogenous RPE proliferation. These first-in-human studies addressed the major risks of PSCs, which are inherently plastic and tumorigenic (Kuroda et al., 2013), by demonstrating safety. Some concern over tumor formation remains, recently heightened by the finding that ESC or iPSC cultures rapidly acquire oncogene mutations during expansion (Merkle et al., 2017). This may occur even in a GMP setting, as an oncogene mutation temporarily halted the Riken clinical trial of iPSC-RPEs prepared for AMD (Garber, 2015). An additional challenge for PSC therapies is that even after extensive differentiation, PSC-RPE progeny remain stalled at the fetal stage of development (Buchholz et al., 2009; Krohne et al., 2012; Leach et al., 2016; Liao et al., 2010), and we do not yet know the extent of maturation achievable subsequent to transplantation *in vivo*. Strategies to produce a mature phenotype in PSC-RPEs are under development (May-Simera et al., 2018). Although challenges remain, the remarkable successes of PSC-RPE transplantation render it the leading RPE replacement strategy.

The RPE-replacement pipeline includes RPE progeny derived from adult RPE stem cells (RPESCs) that are normally present in the native RPE layer. Adult RPESCs have limited proliferative potential and do not form tumors (Salero et al., 2012). They are poised to produce RPE progeny that closely resemble native RPE cells (Blenkinsop et al., 2015). Preclinical transplantation of RPESC-RPEs revealed that an intermediate RPE progenitor stage is significantly more effective at vision rescue than either more or less differentiated RPE progeny (Davis et al., 2017). In this regard, RPESC-RPEs are like other CNS cells for which the progenitor stage engrafts and repairs more effectively (Brundin et al., 1986; Ganat et al., 2012; Warrington et al., 1993). In addition to improved vision rescue in animal models, the use of progenitor-stage progeny reduces manufacturing time and expense.

While several groups are pursuing clinical development of an RPE cell suspension that allows integration into the host RPE monolayer, an alternative approach is to implant stem cell-derived RPE growing on a bioengineered scaffold, which can improve stability and maintain cell polarization (Diniz et al., 2013; Hsiung et al., 2015; White and Olabisi, 2017). Recent reports of ongoing clinical trials (NCT01691261 and NCT02590692) indicate good safety for surgical implantation of RPE-containing scaffolds (da Cruz et al., 2018; Kashani et al., 2018) (Figure 2). Specialized delivery devices have been developed to inject patches of cell-containing scaffolds, which are typically laid on top of the existing RPE layer. These devices decrease the size of the retinotomy (the cut made in the retina for delivery) and thus reduce related surgical complications and enable precise positioning (Fernandes et al., 2017; Stanzel et al., 2012). Additional surgical approaches for transplantation into the subretinal space include a unique trans-choroidal cell delivery instrument (Ho et al., 2017).

Immune reactivity is an important consideration in the success of RPE transplantation. Studies using patient-derived iPSC-RPEs demonstrate the feasibility of autologous transplantation (Mandai et al., 2017b); nevertheless, most approaches are using allogeneic donor cells. The special immune privilege of the eye is due in large part to normal RPE functions, including formation of the blood-retina barrier and production of immunomodulatory cytokines, both of which may be perturbed by RPE disease, RPE cell loss, and surgical trauma during transplantation (Streilein et al., 2002; Xian and Huang, 2015). Clinical trials with allogenic PSC-RPEs use systemic immune suppression for 3–12 months post-transplantation; a limited immune suppression period is thought to provide time for ocular immune privilege to be re-established. The promise of autologous RPEs from iPSCs, which are hoped to be immune-compatible to the patient, is real, but currently limited by the practical expense of manufacturing an individual-use iPSC-RPE product. In the future, HLA-modified cells or multiple banks of stem cell-derived RPE manufactured from PSC or adult RPESC sources to enable HLA matching may improve RPE graft survival.

The RPE supports the overlying retina by performing a wide spectrum of functions, such as phagocytosis, visual pigment processing, fluid and ion transport, and polarized secretion of cytokines. While subretinal RPE transplantation aims to replace these multiple functions, transplantation of non-RPE cells has been pursued with the rationale that they may counter disease through trophic factor secretion. Clinical trials with subretinal umbilical cord stem cells (NCT01226628), subretinal neural stem cells (NCT01632527), and intravitreal RPCs (NCT02320812) are based on this trophic support mechanism of action. However, delivery of non-retinal cells to the subretinal space raises concerns regarding possible complications, given the delicate, specialized physiology of the target tissue. A significant unknown for each approach is whether the transplanted cells will survive, adapt to, and possibly enhance the disease-damaged subretinal environment, or whether biochemical reconditioning of the environment will be necessary for successful cell engraftment (Sugino et al., 2016).

We are now at an exciting point with clinical results emerging from several stem-based trials around the world, through which we have the opportunity to learn about the strengths and weaknesses of different study elements. Finally, beyond transplantation, stimulation of endogenous adult RPESCs present in the eye to produce new, autologous RPE *in situ* without surgery is an exciting future direction.

Bioengineering Approaches to Tissue Regeneration and Stem Cell Therapy for the Eye

Combining stem cell products with bioengineering offers great potential for tissue and organ repair, while raising new challenges to enter the clinical mainstream (Stevens and Murry, 2018). As shown in Figure 3, bioengineering approaches can benefit ocular regenerative medicine by providing (1) scalable and modular 3D cell culture systems for cost-efficient stem cell expansion and/or differentiation; (2) stem cell isolation and purification; (3) biomanufacture and bioassembly of functional eye tissues, including 3D printing; (4) delivery vehicles for better

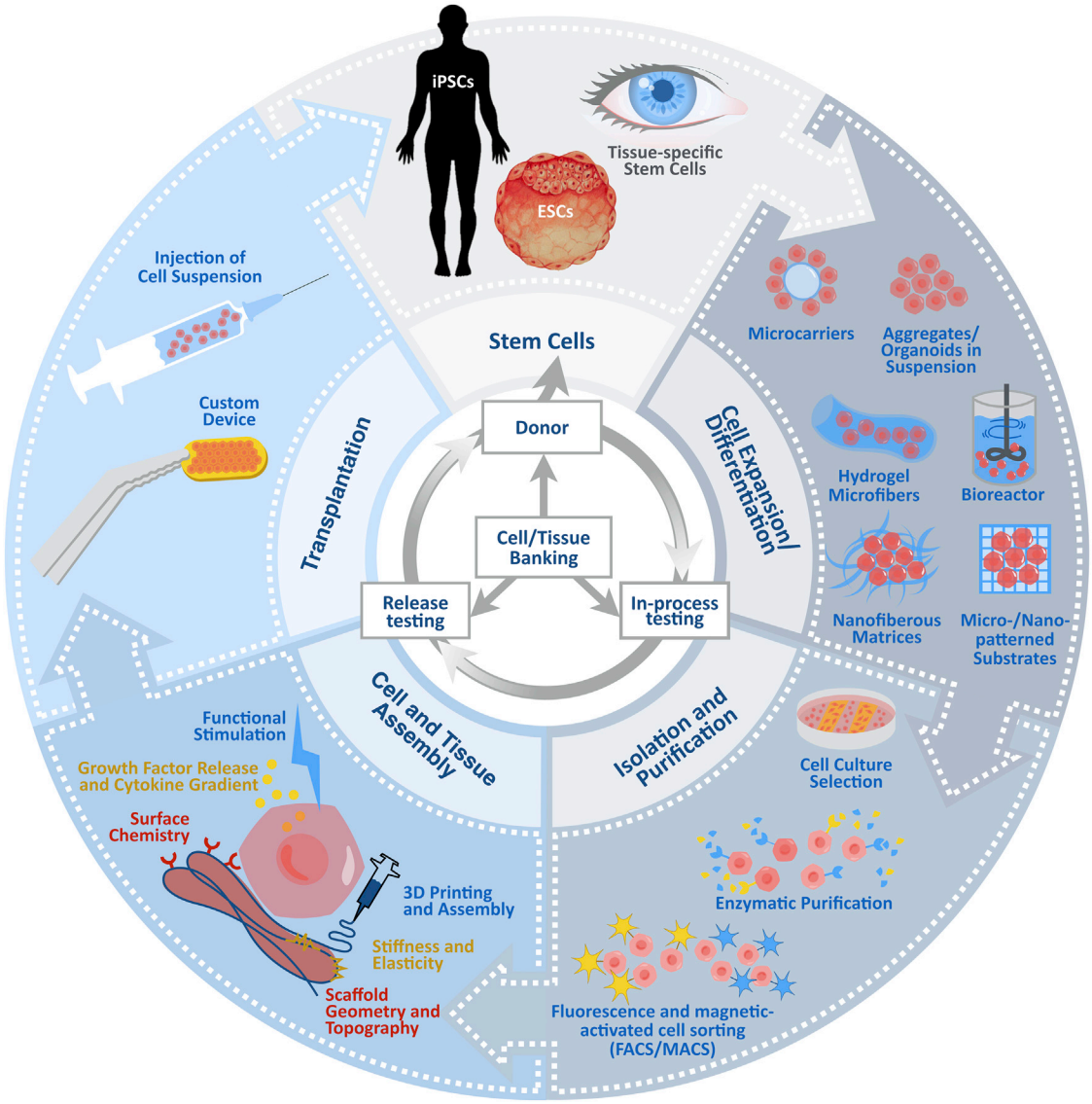

Figure 3. Bioengineering Applied to Cell and Tissue Manufacture and Delivery

Each type of stem cell presents unique manufacturing, regulatory, and clinical challenges that may benefit from approaches depicted in the outer circle of the schematic. The inner circles depict goals common to all stem cell products, including achieving manufacturing standards and testing appropriate for each clinical stage, and efficient manufacturing and banking; for example, using cryopreservation methods that enable easier distribution to clinical sites and flexibility in administration. PSCs readily expand but may be challenging to differentiate appropriately into desired cell types at acceptable purity, while adult stem cells expand less readily but are generally committed to differentiate into specific progeny. A more limited supply of cells from adult sources means multiple cell banks must be manufactured, with challenges associated with comparability testing. Integrating bioengineering approaches can aid each manufacturing step and has the potential to develop complex 3D products to replace key elements in a tissue or organ.

in vivo integration; and (5) engineered tissue constructs for *in vivo* implantation and functional tissue regeneration. Cutting-edge biomaterials with gradients of biomacromolecules such as trophic factors, with tunable mechanical properties or with micro-/nanopatterned topography, have great potential to improve production of ocular tissues or to activate stem and progenitor cell populations present in the eye to encourage endogenous repair.

A variety of bioengineered implants designed to promote corneal regeneration have been tested in preclinical and clinical studies (Griffith et al., 2016). These include limbal epithelial cells on amniotic membranes (Sangwan et al., 2011), corneal stromal cells on polyglycolic acid (PGA) fibrous scaffolds (Hu et al., 2005) or silk substrates (Ghezzi et al., 2017), and carrier-free corneal epithelial and endothelial cell sheets generated on a thermo-responsive polymer (Umemoto et al., 2013). Advances in production of lens progenitor cells and lentoid bodies from human PSCs (Yang et al., 2010) offer plentiful cells that could be combined with bioengineering strategies such as use of sacrificial gelatin bead-hydrogel templates to create lens culture chambers (Wang et al., 2017), and support production of a reproducible lens with desired curvature and mechanics. Primary TM

cells have been cultured on synthetic, microfabricated, well-defined porous SU8 scaffolds to achieve an *in vivo*-like structure and outflow that responds to applied drugs as expected (Torrejon et al., 2016).

Over the past few years, we have come to appreciate the self-assembly properties of stem cells. Pioneering work in the eye showed 3D retina, RPE, and lens-like structures from mouse ESCs (Eiraku et al., 2011; Hirano et al., 2003). Using human PSCs, formation of optic cup and stratified retina was achieved (Nakano et al., 2012). Extending 3D culture to several months yields multi-cell-type retinal organoids including later-born photoreceptor progenitor cells and layers of differentiated photoreceptors that can develop outer segment structures (Lakowski et al., 2018; Wahlin et al., 2017). The differentiation of retinal organoids can be accelerated or enhanced by hypoxia (Chen et al., 2016) or bioreactor use (DiStefano et al., 2018). Organoids may prove valuable for producing specific retinal cell types or 3D retinal structures for transplantation. This is particularly important for conditions such as geographic atrophy, an advanced form of dry AMD in which RPE, neural retina, and the underlying choroidal vasculature degenerate in growing atrophic patches. Retinal multi-layer sheet transplant surgery has advanced (Seiler and Aramant, 2012) and preliminary studies with hESC retinal grafts show evidence of maturation in a primate model (Shirai et al., 2016), although some engrafted cells produced rosette-like structures, previously seen upon fetal retinal progenitor transplantation. We envision that incorporating bioengineering approaches may better preserve retinal layering and integration *in vivo*. At the vanguard, adult RPESC- or PSC-RPE cells cultured with natural or synthetic scaffolds show key features of RPE monolayers that are preserved after transplantation in animal models, among which PLGA, parylene thin film, and polyester membranes are being developed for clinical studies (da Cruz et al., 2018; Kashani et al., 2018; Song and Bharti, 2016; Stanzel et al., 2014; Tian et al., 2017). Bringing together appropriately layered RPE with multi-layered neural retina is important for proper development and function of both, and represents a significant future challenge, as does connecting the output RGC axons with the brain via a rebuilt optic nerve. These challenges will undoubtedly require the combined creative forces of stem cell research and bioengineering.

Challenges in Bringing Ocular Regenerative Therapy to Patients

Across these efforts to repair diverse ocular tissues, we see common hurdles. Immune response is a major issue for allogeneic cell transplants, which can be alleviated by HLA matching. This has spurred production of HLA-defined banks of iPSCs (de Rham and Villard, 2014) and HLA-engineered iPSCs with potential as universal donor lines (Gornalusse et al., 2017). Developing efficient and scaled clinical manufacturing is critical to ensure that the cell product can be delivered to patients safely, effectively, and at a cost that is reasonable, appropriately reimbursed, and economically responsible. For any stem cell source, even those with great expansion potential, cell manufacture will at some point need to be repeated, and ensuring that each batch is comparable represents a significant challenge (NAS, 2017). Identification of the Critical Quality Attributes, i.e., characteristics that ensure the desired product quality, is difficult for cell products, which are highly complex, change over time, and can be affected by minor changes in the many molecules used in manufacturing. Currently, cell products are defined by a limited number of markers to reflect identity, purity, and potency, but there is recognition that this level of characterization may be inadequate (Barazzetti et al., 2016). We look ahead to improved phenotyping methods, such as single-cell-level characterization, that better define cell product composition. To enter the clinic, testing in animal models is essential. Disease models are often problematic, however, as they don't necessarily predict outcomes in patients. Animal safety testing for toxicity and tumorigenicity typically is done under good laboratory practice (GLP), with hundreds of animals, extensive live phase observation, and histological analysis resulting in a significant financial barrier of multimillion dollar costs. Here we ask, could iPSC-based *in vitro* disease eye models, which are advancing in sophistication (Song and Bharti, 2016), be useful as substitutes for animal testing? Throughout the process of bringing a cell therapy to the clinic, a close working relationship with the regulatory authority is advantageous and encouraged by bodies such as the FDA in the United States, in order to ensure that an Investigational New Drug application for clinical trial has the best chance of success. Once clinical evidence has been obtained, several countries have implemented mechanisms to accelerate regenerative therapy development, such as the Regenerative Medicine Advanced Therapy (RMAT) designation in the United States. Japan pioneered accelerated conditional approval of cell therapies that allow reimbursement, contingent on further gathering of positive post-market clinical outcomes. On the one hand, these mechanisms to accelerate therapy development are welcome, given the great unmet need, but we must ensure that the quality and scrutiny of post-approval data are high to eliminate ineffective therapies from the healthcare marketplace (Smith and Brindley, 2017). Many groups are moving through this process with new ocular regenerative medicine products, and while arduous, the shared goal is to ensure safe and effective therapies for eye disease. As a tragic reminder of what can happen if this process is side-stepped, inappropriate use of cell products in "patient-funded research" by stem cell clinics that skirt appropriate regulatory oversight has led to devastating outcomes; for example, intraocular transplantation of liposuctioned fat-derived cells has blinded several patients (Kuriyan et al., 2017).

Conclusion

The exciting advances for diverse ocular indications across different eye tissues reveal a promising pipeline of clinical translation (Figure 2). We ask, "How far are we from creating not just single eye tissue modules, but modules that function together, and perhaps even a whole eye?" PSCs can be coaxed down multiple ocular lineages, producing structures with corneal, lens, and retinal zones (Hayashi et al., 2016, 2017). Progress is being made in whole-eye transplantation with a patent vasculature, although a certain challenge is how to connect the neural elements so that the transplanted eye functions completely (Bourne et al., 2017). Our ability to even contemplate this question is indebted to studies of development and regeneration in model organisms and advances in human stem cell systems. What was once unfathomable now comes into focus as challenging yet tractable. This audacious goal is being pursued by

teams of scientists, engineers, and clinicians across disciplines through concerted, collaborative efforts. When Charles Bonnet lost his sight, he relied on close collaborators and cultivated a large network of scientists around the world with whom he communicated regularly, writing hundreds of letters each year. It is obvious from his collected writings (Bonnet, 1779) that he valued and respected the contributions of others, and that he understood that science progresses best by working together: "We can persuade ourselves that the more we investigate deeply, the more uses will emerge…. The truths become brighter as one advance builds on another."

SUPPLEMENTAL INFORMATION

A video abstract is available at https://doi.org/10.1016/j.stem.2018.05.013#mmc1.

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DECLARATION OF INTERESTS

G.P.: consultant of J-TEC, Gamagori, Japan, and founder and R&D director of Holostem Terapie Avanzate, Modena, Italy; patent: Reconstructed laminae of human epithelium corneae and method of producing the same, US6610538B2. S.T. and J.H.S.: patent: Retinal pigment epithelial stem cells, US8481313B2. K.Z.: patent application: Lens regeneration using endogenous stem/progenitor cells, WO2017100463A1. J.F.: patent: Inhibition of proliferation and fibrotic response of activated corneal stromal cells, US8815946B2; patent application: Bioengineered human corneal stromal tissue, WO2011109712A2; patent application: Trabecular meshwork stem cells, US20120237485A1. Y.X.: patent: Bioengineered human trabecular meshwork for biological applications, US9506907B2; patent application: Use of vascular cells to create the conventional outflow tract, WO2017130178A3. J.L.G.: patent: Magnetic cells for localizing delivery and tissue repair, US9078932B2; patent application: Use of encapsulated cell therapy for treatment of ophthalmic disorders, US20180055766A1. R.R.A.: patent application: Cell surface biomarker panel for the isolation of human photoreceptor cells, UK1703058.6.

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