Ocular regeneration by stem cells: present status and future prospects

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Background: Advances in the stem cell field provide much hope for the use of these cells in the regeneration of ocular tissue damaged by diseases for which no treatments are yet available. Here, we discuss the current status and limitations on the application of stem cells to ocular therapies, and consider the future prospects for their use in the restoration of vision.

Source of data: The review summarizes the achievements to date and the present areas of stem cell investigations in the ophthalmic field, based on a literature search and knowledge gained by the authors’ work in the subject.

Areas of agreement: Owing to its accessibility, the cornea constitutes an easy anatomical target for stem cell regeneration. On this basis, limbal epithelial stem transplantation is the only ocular cell-based therapy already in use in the clinical setting.

Areas of controversy: Regeneration of the retina, a less accessible and complex neural tissue, currently constitutes a major challenge. Investigations into the potential use of stem cells for retina regeneration have generated variable data and no therapies have yet been designed for human treatments.

Growing points: Despite the present limitations, it has been progressively accepted that various stem cells may have potential use for the development of cell-based therapies to restore retinal function.

Areas for research development: There is need to understand the cell requirements and environmental conditions that may promote functional integration and long-term survival of stem cells within the diseased retina. At present, this constitutes a major area of research.

Keywords: retinal stem cells/Muller stem cells/limbal epithelial stem cells/retina/cornea
Introduction

Recent developments in the stem cell field have widened the prospects of applying cell-based therapies to regenerate ocular tissues that have been irreversibly damaged by disease or injury. In the clinical setting, stem cell transplantation to repair and regenerate human corneal epithelium has been successfully used for several years.\textsuperscript{1} Understandably, the corneal surface provides an easy anatomical site in which stem cell-based therapies can be implemented without using invasive methods. This is not the case with less accessible and complex tissues responsible for visual function, such as the neural retina and the retinal pigment epithelium (RPE), for which many problems to establish such therapies remain to be solved.

Different parts of the eye derive from different types of embryonic tissue: the neurosensory retina and the RPE arise from the neuroectoderm, the corneoscleral and uveal tunics develop from the mesoderm and the lens derives from the ectoderm.\textsuperscript{2} Therefore, regeneration of the different ocular tissues poses a significant challenge due to their cellular diversity and to the organization of neural networks found in the retina and the optic nerve. In this context, the feasibility of regeneration may depend on the complexity of the tissue to be repaired. For example, regeneration of RPE may provide a relatively easy target for stem cell-based therapies as this constitutes a single cell monolayer. The neural retina, however, may require more complex protocols for its regeneration due to its multilayer structure of specialized neurons and glial cells. During early disease, regeneration of a single neural cell type may prove more feasible than in late disease when all types of neurons have been compromised. Whichever the case, retinal repair and regeneration may not only require a better understanding of the molecular mechanisms that promote stem cell differentiation into functional neurons and glia, but also of the requirements for successful migration, integration and survival of the transplanted cells. This is of special importance as a retina in need of repair may have lost the developmental cues that allow permissiveness for neural integration, and it is most likely to exhibit pro-inflammatory and gliotic barriers that prevent successful regeneration by transplanted cells. Here we summarize the achievements to date in the field of stem cell therapies to treat ocular disease, and draw attention to the existing hurdles that need to be overcome in order to establish successful cell-based treatments to restore visual function.
Retina

Stem cells and retinal development

It has been known for a long time that fish and amphibians have the ability to regenerate neural retina throughout life, and that stem cells capable of regenerating the retina in these species are located at the ciliary margin. The presence of stem cells in the retinal ciliary margin has also been documented in avians and small mammals, and more recent studies have identified retinal stem cells in the same region of the human eye. In addition, Müller glial cells have been shown to have the potential to regenerate new neurons in response to acute damage in the newly born chick and rat retina. That Müller glia exhibit neurogenic characteristics in the adult eye have been further supported by the unequivocal demonstration that in the adult zebrafish, Müller glia form the retinal stem cell niche and that they are able to generate retinal stem cells after retinal injury. Interestingly, more recent investigations have identified a population of Müller glial cells with stem cell characteristics in the adult human retina (illustrated in Fig. 1), which suggests that the human retina, like in the zebrafish, may have some potential for regeneration.

During development, Müller glia and retinal neurons share a common progenitor that is multipotent at all stages of retinal histogenesis. This evidence derives from examination of the progeny of a single mouse retinal progenitor cell transfected with a retrovirus, which generated clones containing up to three types of neurons, whereas others contained a combination of neurons and Müller glia, Müller glia alone or a single type of neuron. Studies of retinal neurogenesis in small vertebrates have shown that the generation of retinal neurons follows an evolutionary conserved sequence, in which retinal ganglion cells, cone photoreceptors, horizontal cells and a population of amacrine cells are born during early stages of retinal histogenesis, whereas bipolar cells, Müller glia and most rod photoreceptors are born during late stages of histogenesis.

Criteria for choosing the source of stem cells for human retinal transplantation

Early experimental studies in the 1980s to regenerate visual function included the transplantation of whole eyes to genetically eyeless salamanders, the implantation of peripheral nerves into adult rat retina and the grafting of embryonic rat retina into a lesion site of adult rat retinae. Since then, several approaches to regenerate retina have been made in various experimental models of retinal degeneration using
stem cells derived from several sources. These include brain-derived stem cells, embryonic retinal progenitor cells, ciliary epithelium and stem cells from the postnatal eye, human embryonic stem cells, umbilical cord tissue cells and mesenchymal stem cells, bone marrow stem cells and Müller stem cells (illustrated in Fig. 2). However, despite intensive research in the field, to date there is no sufficient evidence for widespread stem cell integration into the retina, long-term graft survival or complete restoration of visual function.

The human neural retina, like any mammalian, is organized into three different nuclear cell layers that harbour six different types of neurons and circa 60 neuronal subtypes. The outer layer is populated by photoreceptor cells (light sensitive neurons); the middle layer (known as the inner nuclear layer) homes bipolar, amacrine and horizontal cells and the innermost layer (known as the ganglion cell layer) predominantly contains ganglion cells and displaced amacrine cells. Upon visual stimuli, all retinal neurons undergo a complex cascade of neural interactions through activation of synaptic pathways which finally lead to ganglion...
cells conveying visual messages via the optic nerve onto the brain. This complex process requires intact axonal connections, which are lost during retinal degeneration, and potential restoration of visual function critically depends on whether transplanted neurons can re-establish the dynamic neural network of the retina. Potential success of stem cell-based therapies may therefore depend on whether grafted cells can differentiate into the different retinal neurons, and whether these neurons can re-establish normal synaptic pathways within the host retina.

Although significant progress has been made in recent years towards the understanding of the molecular mechanisms that determine retinal cell specification in fish, amphibians and small vertebrates, and there is much overlap in the expression of progenitor markers in the developing human retina with that of other species, it is well recognized that there is still much to be learned about the signals that lead to specific neuronal development in the human eye. In this context, although different types of stem cells can be potentially used for regeneration of the diseased human retina, it may be more appropriate, as suggested by recent studies to use adult cells that have already undergone crucial developmental stages to make them committed to become retinal neurons. This would bypass extensive procedures that may be needed for in vitro differentiation of embryonic, haematopoietic, mesenchymal or brain stem cells into retinal progenitor cells, especially since we do not know in depth the molecular pathways needed for such differentiation. In addition to the state of differentiation of stem cells potentially used for

**Fig. 2** Müller stem cell transplantation in experimental models of retinal degeneration. Following 2 weeks after transplantation of adult human Müller stem cells into the subretinal space of the dystrophic RCS rat, only a small number of transplanted cells (green) is able to migrate and integrate into the retina. ONL, outer nuclear layer (photoreceptor cells); INL, inner nuclear layer. Red staining indicates the expression of recoverin, a protein expressed by photoreceptor cells.
transplantation, special consideration should be given to the environment in which stem cells are to be transplanted. Since the diseased retina is not a permissive milieu in which stem cells could easily integrate, but damaged tissue in need of repair, it also may be necessary to design adjuvant therapies to modify the extracellular matrix to facilitate functional stem cell integration. As evidence for the existence of ‘adult’ retinal stem cells in the human eye has been presented, it is clear that these cells may have advantages in terms of immediate clinical application, safety and feasibility for transplantation.

Characterization and isolation of retinal stem cells

Retinal stem cells can be isolated and induced to proliferate in vitro from the foetal and adult eye by enzymatic dissociation, followed by culture in the presence of growth factors such as fibroblast growth factor and epidermal growth factor. Several markers have been identified that allow the characterization of these cells, including nestin and βIII tubulin, as well as various transcription factors and proteins expressed by retinal stem cells during in vivo development. Some of the most important factors known to characterize neural retina stem cells include: (i) Sonic hedgehog protein (Shh), which in early development promotes differentiation of progenitor cells into ganglion cells and acts as a signalling molecule; (ii) Pax6, a regulatory factor that promotes multipotency of retinal progenitor cells; (iii) Basic helix-loop-helix (bHLH) transcription factors, such as Math 5 and NeuroD, which upon activation drive progenitor cells towards the ganglion cell and amacrine lineages, respectively; (iv) Chx10, one of the earliest markers of the developing retina, which is required for retinal cell proliferation and formation of bipolar cells and (v) Sox2, a transcription factor found in early neurogenesis, which is down-regulated as cells differentiate and migrate to the different retinal cell layers. Expression of Sox2 has also been shown in precursors of the human foetal retina and constitutes an important marker of Müller stem cells in the adult human eye. Other factors recognized to drive differentiation of retinal progenitors include the receptors for fibroblast growth factors (FGF-R) and epidermal growth factor (EGF-R), which play an important role in neural and glial differentiation.

Prevalent diseases that may potentially benefit from stem cell therapies

Neural cell death is the ultimate cause of blindness as a result of retinal degenerative diseases, including age-related macular degeneration
(AMD), proliferative diabetic retinopathy (PDR), end-stage glaucoma, retinitis pigmentosa (RP), proliferative vitreoretinopathy and inherited retinal diseases. AMD is the leading cause of blindness in the western world, affecting between 20 and 25 million people worldwide.\(^{24}\) It is estimated that between 200,000 and 300,000 individuals in the UK are registered as blind or partially sighted as a result of AMD.\(^ {24}\) In England and Wales, DR is the most common cause of blindness among adults aged 16–64, afflicting more than 35% of individuals after 20 years of diabetes.\(^ {25}\) At present, treatments with anti-angiogenic factors, laser photocoagulation and surgical treatments are only able to delay visual loss in these patients, but in a long term, it is impossible to prevent blindness finally occurring. On this account, the only hope for restoration of sight is the development of stem cell-based therapies to replace the damaged neurons and that could potentially integrate into the remaining neuronal network.

**Retinal pigment epithelium**

The RPE constitutes a monolayer of pigmented cells attached to the inner surface of the Bruch’s membrane. It is directly positioned over the photoreceptor outer segments and plays an important role in retinal homeostasis.\(^ {26}\) RPE dysfunction occurs in severe retinal diseases such as RP and AMD,\(^ {27}\) for which stem cell transplantation to regenerate RPE cells may constitute a promising approach for treatment of these diseases.

Major advances in the development of stem cell therapies to regenerate RPE have been made during the last few years, with the findings that human embryonic stem cells provide a well-characterized and reproducible source of RPE that could potentially be used for human clinical studies.\(^ {28}\) Transplantation of RPE derived from human embryonic cells have been transplanted in animal models of RPE degeneration and has been shown to improve photoreceptor cell survival.\(^ {29}\) On this basis, extensive research on the potential of stem cells to regenerate RPE cells for transplantation to patients with RP and AMD is being actively pursued by several groups. Human RPE cells derived from embryonic cells have not yet been used in the clinical setting, but with the technology and knowledge available at present, it would not be long before these cells can be applied to human therapies. However, as with any other cell therapy, generation of these cells need to be performed under therapeutic conditions, and their safety and immunogenicity need to be examined before they can be applied to human treatments.
Cornea

Stem cell maintenance of corneal epithelium

The transparency of the cornea and therefore visual acuity is dependent upon the integrity and functionality of the outermost layer, the epithelium. Corneal epithelial stem cells reside at the junction between the cornea and neighbouring conjunctival epithelium in a region known as the limbus. These cells, known as limbal epithelial stem cells (LESC), are responsible for maintaining the corneal epithelium throughout life. When a stem cell in the limbus divides in response to homeostatic cell loss, it gives rise to transient amplifying cells which migrate, proliferate and differentiate to populate the entire corneal epithelium. Evidence for the corneal limbus being the source of an adult stem cell population was first suggested by observations in the early 1970s that pigment in the epithelium of heavily pigmented eyes migrated in lines from the limbus to the central cornea in healed eccentric epithelial defects. The existence of slow-cycling limbal epithelial basal cells was later confirmed by observations that cells localized at the limbus retained tritiated thymidine label for long periods of time, a characteristic of slow cycling stem cells. Detailed examination of the corneal limbus niche has recently shown the existence of characteristic topographical structures associated with concentrated deposits of LESCs, which predominantly occur in the superior and inferior limbus. It is interesting to speculate that these regions, which are normally covered by the upper and lower eye lids, have evolved to provide a degree of protection to the stem cells from environmental insults such as ultraviolet light.

Present approaches to corneal stem cell therapy

Ocular surface failure resulting from LESC deficiency can occur as the result of primary (inherited eye disease) but more commonly as the result of acquired factors, including chemical burn injury, contact lens keratopathy, chronic limbitis, limbal surgery or Stevens–Johnson Syndrome. When LESC deficiency occurs the neighbouring conjunctival epithelium, which is normal prevented from encroaching in the corneal surface by the LESCs, migrates to cover the ocular surface in a process known as conjunctivalization. When this happens, the patient can experience repeated epithelial erosion, blood vessel growth across the normally avascular cornea, inflammation, pain and eventual loss of sight. Previous clinical approaches to the treatment of LESC deficiency included autologous transplantation of limbal tissue from the
contralateral healthy eye to the diseased eye. Although successful, this procedure carries the risk of creating LESC deficiency in the donor eye. Alternatively, limbal tissue allografts have been performed using living related or cadaveric donor material. However, due to the abundance of HLA-DR antigens and Langerhans cells in the graft, patients must undergo long-term systemic immunosuppression.

In 1997, the first successful cultured stem cell therapy for LESC failure was reported in two patients with chemical burn injuries. The procedure involved the isolation and *ex vivo* expansion of autologous LESC from a 1 to 2 mm² limbal biopsy for transplantation. The clinical outcome was promising with both patients experiencing improved vision for at least 2 years (illustrated in Fig. 3). Since then a number of centres around the world have employed the technique using a variety of different cell culture protocols and carrier systems for transferring the cells to the patient, including the use of cultured LESC from allogeneic cadaveric donors.

The various methods for LESC therapy production and reported clinical outcomes have been comprehensively reviewed recently. The overall success rate for the combined results of cultured autologous and allogeneic LESC therapy treatments is ~70%. The caveats to consider when interpreting the reported data include the subjectivity of assessment in some studies, the differing aetiologies of LESC failure, the cell culture protocols adopted, the myriad of previous ocular surgical interventions and the pre and post-operative management. Despite these limitations, the data suggest that cultured LESC therapy is a beneficial adjunct for the treatment of ocular surface failure.

At present, there is much research centered around the evaluation of alternative stem cell sources for corneal stem cell transplantation, including embryonic stem cells. One autologous cell source of
significant interest is the oral mucosal epithelial cell (OMEC) or buccal keratinocyte population. The first reports on the use of cultured OMECs for the treatment of LESC deficient patients initially showed promising results, but subsequent studies have shown that neovascularization can occur in some patients after transplantation. This indicates that there is still a lot to be understood about the interactions between the corneal stem cell and its niche environment with respect to regulation and function.

**Characterization of LESC**

Despite the proliferative capacity of LESC, conclusive identification of their specific markers has not yet been achieved. A number of ‘negative limbal basal cell markers’ including absence of cytokeratins 3 and 12, connexins and gap junctions have been proposed, along with ‘positive limbal basal cell markers’ including co-localization of vimentin and keratin 19, immuno-localization of the glycolytic enzyme alpha-enolase, high levels of expression of epidermal growth factor (EGF) receptors and expression of the transcription factors p63α, PAX6 and Shh. Further investigations into the expression of specific markers by LESC may aid in the identification of these cells and facilitate the diagnosis of LESC failure.

**Clinical limitations and accessibility to LESC transplantation**

The main clinical limitation to the use of cultured LESC therapy is the availability of autologous donor tissue. Although cultured allogeneic cells may be used in combination with systemic immuno-suppression, there are indications that donor allogeneic LESCs do not survive in the long term, and therefore immuno-suppression may not be required beyond a period of 9 months. This was suggested by evidence that donor DNA cannot be detected after these period, yet, patients maintain a corneal epithelium that appears to be of autologous origin. These observations raise the interesting question about the mechanism of cultured LESC therapy efficacy. It is possible that bone marrow stem cells may be the source of the newly regenerated corneal epithelium, or that the allogeneic cells may in some cases provide a permissive environment for previously quiescent autologous LESC to regain function. Key to the outcome of cultured LESC therapy in the cornea appears to be the pre-operative management of inflammation and infection both of which will compromise the efficacy of the treatment if not fully controlled. In order to prospectively identify those
patients who may benefit from cultured stem cell transplantation, not just in the eye, clearly defined objective parameters of pre and post-operative assessment must be developed to enable proper comparisons between studies.

Provision of cell therapies, particularly customized autologous treatments will remain expensive until the issues around scale-up and commercialization have been addressed. Therapies at present conducted under research protocols (which still need to meet the provisions of the EU Tissue and Cells Directive) are produced by highly specialized research-driven facilities and hence are not widely available to the population. The clinical follow-up time required to critically evaluate cell therapy safety and efficacy in patients is time-consuming and difficult to do in any volume in regular clinics. However, it is anticipated that in the future this will change as new innovations deliver more efficient cell therapy production protocols and robust methods to assess clinical outcome.

**Other progenitor cells in the eye**

Recent evidence shows that the conjunctiva is also a source of stem cells. A detailed *in vitro* study of the clonogeneic properties of the ocular surface epithelia revealed a uniform distribution of putative stem cells in the bulbar and fornical conjunctiva.\(^4^0\) It showed that conjunctival epithelial and goblet cells derive from a common progenitor with high proliferative capacity that gives rise to goblet cells at least twice during their life cycle and at precise moments prior to senescence. Conjunctival epithelial cell progenitors which can give rise to goblet and non-goblet cell phenotypes have been cultured *in vitro* on amniotic membrane and have been shown that like the LESC, are slow cycling and label retaining. Cultured conjunctival epithelial cells, thought to contain a proportion of stem cells, have been used to successfully treat patients with ocular surface damage.\(^4^1\)

RPE and iris pigment epithelium from adult murine eyes, as well as choroid and scleral cells from adult human eyes, have been shown to de-differentiate *in vitro* to express antigens of retinal neurons. However, the capacity of long-term cell renewal and evidence that they constitute a true population of stem cells has not yet been proven. In addition, there is some evidence that LESC exhibit neural plasticity,\(^4^2\) raising the possibility of exploring these cells to generate retinal neural progenitors.

Other recent studies have identified a population of keratocyte progenitors in the corneal stroma of the murine and human eyes. Progenitor cells derived from the corneas of C57BL6/J mice are able to
form spheres, express Pax6 and maintain their keratocyte phenotype after several passages.\textsuperscript{43} Similarly, stromal cells isolated from the region surrounding the human corneal limbus express Pax6 and the ABCG2 transporter protein observed in many adult stem cells. Upon culture with FGF2, they express keratocyte markers; and following differentiation, they acquire chondrogenic and neurogenic characteristics,\textsuperscript{44} indicating a multipotent ability of these cells. These findings suggest that keratocyte progenitors have potential use for cell-based therapies to treat many corneal diseases, and exploration of their capabilities would further advance stem cell therapies to treat ocular surface disease.

**Regulatory issues affecting established stem cell therapies**

In order to produce and deliver cell therapies in Europe, it is now necessary to comply with the provisions of the EU Tissues and Cells Directive. In the UK, this is governed by the Human Tissue Authority (HTA). The purpose is to protect patient safety and to ensure that the best quality products are used in clinical trials and routine practice. This involves producing the therapy in a controlled clean room environment under an extremely stringent quality management system akin to the principles of Good Manufacturing Practice used by the pharmaceutical industry.\textsuperscript{45} This poses great practical challenges when cultivating cells in a dynamic living system compared with drug manufacture, and would have huge financial repercussions in the health service if cell therapies were to be widely established to treat ocular disease.

**Conclusions**

Stem cell-based therapies for ocular repair and regeneration constitute a major hope for the restoration of visual function in individuals whose ocular tissue has been irreversibly damaged by disease or trauma. At present, the only cells with well-recognized clinical application in the ophthalmic field are the LESC for corneal repair. Experience gained from this approach would potentially help with the design of stem cell-based therapies to regenerate other ocular tissues, particularly the retina. There is much speculation on the capability of newly discovered sources of retinal progenitors, but before establishing any new therapy, we must understand the real potential of each cell source to regenerate retinal neurons. In addition, there is the need to carefully address important issues that are at present precluding the
establishment of human retinal therapies. Problems to be solved comprise the standardization of isolation and expansion methods to preserve stem cell phenotype, the identification of requirements for neural differentiation and preparation of cellular scaffolds for grafting, the modulation of the extracellular matrix to promote cell migration and neural synapses, the prevention of immune rejection and tumour development and, very important, the identification of conditions that maintain graft survival. Despite many practical problems, there is general optimism among the medical and scientific community that stem cell-based therapies to restore visual function could become a reality in a non-distant future.

References