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Stem Cell Based Therapies for Glaucoma

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1. Introduction

Glaucoma remains one of the leading causes of blindness worldwide. In England and Wales glaucoma is a major or contributory factor for 12-14% of all registrations for blindness and partial sight, second only to macular degeneration (Bunce et al., 2010). The worldwide burden is more significant, with glaucoma being the second leading cause of global blindness after cataract (Resnikoff et al., 2004). It has been estimated that 60.5 million people worldwide would be affected by glaucoma by 2010, with the figure expected to rise to 80 million by 2020 (Quigley and Broman, 2006).

Current treatments for glaucoma comprise the lowering of intraocular pressure by eye drops, laser procedures or drainage surgery. However, as implied by the statistics above, many patients experience significant visual loss due to degeneration of retinal ganglion cells (RGCs) despite the advances in the treatments currently available. The need for novel therapies exists for such patients, in particular those with end stage glaucoma, where the maintenance of a small number of surviving RGCs may yet permit a reasonable quality of life (Much et al., 2008). Stem cell therapies developed in the laboratory and translated to clinical practice provide an exciting and realistic hope for those affected by degenerative retinal diseases including glaucoma. This chapter will discuss three mechanisms by which stem cell therapies may potentially offer hope to patients with end stage glaucoma, namely local RGC replacement, optic nerve regeneration and stem cell mediated neuroprotection.

2. Sources of stem cells

Stem cells are characterised by their capacity for unlimited self-renewal and ability to differentiate into different cell types. The term progenitor cell is often applied to multipotent cells with a capacity for self-renewal, however this chapter will use the term stem cell to encompass all progenitor and precursor cell types.

An ideal candidate for developing stem cell based therapies would be readily available, easy to expand in culture, possess an acceptable long term safety profile and be autologous in nature, in order to avoid the need to modulate the host immune response and prevent rejection. Unfortunately a cell type that fulfils all these criteria remains elusive, however current research is directed towards a limited number of cell types which themselves exhibit certain advantages or disadvantages. Such cell populations may be sourced from three broad categories – embryonic or foetal tissue, adult tissue and reprogrammed cells (Figure 1).

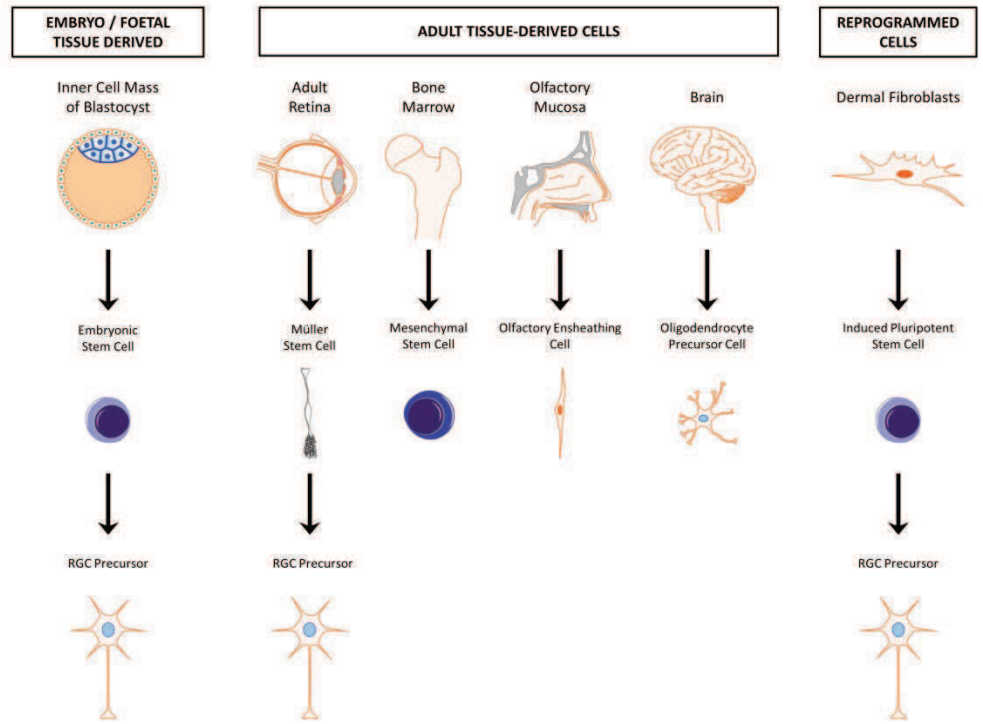

Fig. 1. Summary of the sources of cells that may be potentially used for cell based therapies in glaucoma. (Figure composed using Motifolio Inc. diagrams)

2.1 Embryonic stem cells

Embryonic stem cells (ESCs) arise from the inner cell mass of the blastocyst, which is formed at about five days after fertilisation in humans. Such cells are often sourced from excess tissue obtained from embryo donations and fertility treatments and have been associated with ethical objections due to controversies regarding the use of such tissue for research. However they possess an unlimited capacity for self-renewal with an ability to differentiate into any of the cell types within the human body (Evans and Kaufman, 1981). ESCs have been proposed as ideal candidates for cell based therapies to treat human retinal diseases, due their capacity to migrate and differentiate into different cell types. ESCs have been differentiated *in vitro* into neurons (Bibel et al., 2004) as well as retinal pigmented epithelium (RPE) (Hirano et al., 2003), but controlling their differentiation has proved challenging. In the absence of appropriate intracellular signals, ESCs appear to differentiate towards a neuronal fate by default (Hemmati-Brivanlou and Melton, 1997), although differentiation into retina specific precursors often involves complex laboratory protocols (Osakada et al., 2009). A drawback of a pluripotent cell type is the risk of teratoma formation by uncontrolled growth of transplanted ESCs (Hentze et al., 2007) which remains a major concern. In addition, safety concerns derived from the observed chromosomal instability of cultured ESCs (Moon et al., 2011) require further investigation.

2.2 Adult tissue-derived stem cells

Adult tissue-derived stem cells offer an alternative for the development of cell based therapies which circumvents the ethical controversies surrounding foetal and embryonic tissue. Up to date, various sources of adult stem cells have been investigated for their potential ability to regenerate or replace retinal neurons which are described below.

2.2.1 Müller stem cells

The concept of central nervous system (CNS) regeneration from glial cells has become more accepted in recent years. Radial glia within the brain have been shown to act as neural stem cells within the developing mammalian nervous system, with the ability to generate both new neurons and glia (Merkle et al., 2004). Müller glia are the radial glia of the retina and have been shown to share a common lineage with retinal neurons and to derive from a common multipotent progenitor (Turner and Cepko, 1987). Studies in zebrafish have demonstrated that the ability of this species to regenerate retina is due to the presence of Müller glia with stem cell characteristics (Bernardos et al., 2007). Pharmacological depletion of the ganglion cell layer has been shown to induce a regenerative response in this species, which is characterised by Müller glial cells re-entering the cell cycle and producing neuronal progenitor cells that repopulate the ganglion cell layer (Fimbel et al., 2007).

Although a capacity for regeneration similar to that seen in the zebrafish has not been observed in higher species, a population of Müller glia with stem cell characteristics has been identified in the adult human retina (Lawrence et al., 2007). These cells express markers of neural progenitors *in vitro* and a proportion of them are able to express markers of mature retinal neurons in response to various culture conditions (Lawrence et al., 2007). Data from our laboratory exploring transplantation of these cells in a rodent model of ganglion cell depletion shows that pre-differentiated cells are able to integrate within the host RGC layer and cause partial restoration of the scotopic threshold response, which is a marker of RGC function in the rat electroretinogram (Singhal et al., 2009).

Such cell lines are easily obtained from cadaveric donor retinae (Limb et al., 2002) and further studies may reveal whether it is possible to obtain patient specific cell lines from peripheral retinal biopsies, leading to the possibility of developing an autologous grafting strategy.

2.2.2 Mesenchymal stem cells

Mesenchymal stem cells are most commonly obtained from bone marrow biopsies and umbilical cord blood and have been considered as candidates for autologous cell transplantation. Pharmacological methods have been used to mobilise haematopoetic stem cells from the bone marrow into the bloodstream to facilitate their harvesting for transplantation (Uy et al., 2008) rather than employing more invasive bone marrow trephine techniques. The mobilisation of mesenchymal stem cells is more difficult than that of haematopoietic stem cells, with several strategies showing promise in animal models (Pitchford et al., 2009). During development mesenchymal stem cells differentiate into bone, cartilage and muscle. However they have been reported to de-differentiate *in vitro* into other cell types including neurons and glia, although at present there is much controversy surrounding this ability (Krabbe et al., 2005). As will be discussed later, this cell type is likely to have a more significant role in neuroprotective strategies rather than neuronal replacement, due to their ability to secrete cytokines.

2.2.3 Oligodendrocyte precursor cells

Oligodendrocyte precursor cells (OPCs) are a type of neural stem cells responsible for the generation of oligodendrocytes during normal development, and for re-myelination of the white matter in the adult CNS (Watanabe et al., 2002). They are the commonest proliferative cell type in the adult CNS (Dawson et al., 2003). OPCs have been reported to exhibit some stem cell characteristics (Nunes et al., 2003) and neuroprotective potential *in vitro* (Wilkins et al., 2001), which have led to investigations into their potential use for stem cell based therapies to treat neurodegenerative conditions including glaucoma.

2.2.4 Olfactory ensheathing cells

Olfactory tissue is unique within the CNS, in that continuous removal and regeneration of tissue occurs throughout life. The sensory axons that project to the olfactory bulb are closely associated with specialised cells known as olfactory ensheathing cells (OECs). OECs are glial cells which lie within the nasal mucosa and olfactory bulb and characteristically ensheathe axons of the olfactory nerve. Transplantation of these cells has been used to support regenerating axons in animal models of spinal cord injury and to restore function (Li et al., 2008). Due to the relative ease by which nasal mucosal biopsies may be obtained, these cells may potentially constitute a source of cells through which autologous transplantation strategies may be developed in the future. There is considerable molecular heterogeneity and functional diversity of OECs with much work still taking place in animal models (Su and He, 2010). Further investigation into the gene expression and cell fate determination of these cells will facilitate the development of more robust protocols to isolate and expand the OEC progenitor/stem cell population within this complex tissue.

2.3 Induced pluripotent stem cells

The characterisation of induced pluripotent stem cells (iPS) cells has created an alternative potential cell source for transplantation in regenerative medicine. Takahashi & Yamanaka (Takahashi and Yamanaka, 2006) demonstrated that by retroviral induction of Oct3/4, Sox2, c-Myc and Klf4, pluripotent stem cell lines could be derived from fibroblast cultures. Further study of these "reprogrammed" iPS cells showed that their biological behaviour was indistinguishable from that of ESCs (Wernig et al., 2007). Subsequent modifications to the original protocol have enabled iPS cell lines to be created without the use of viral vectors (Okita et al., 2008) and without induction of the oncogene c-Myc (Nakagawa et al., 2008) which may be associated with an increase in tumorigenesis. However before such cells can be used in human therapies, safety concerns regarding the effect of the reactivation of pluripotency, alterations in target cells and characterisation of these cells need to be addressed (Jalving and Shepers, 2009).

3. Potential of stem cells for retinal ganglion cell replacement

One of the strategies to restore vision in glaucoma patients after RGCs have been lost or irreversibly damaged is their functional replacement by autologous or heterologous transplantation.

It is generally accepted that damage to the neural retina during glaucoma is restricted to the impairment of function and subsequently degeneration of RGCs (Kerrigan-Baumrind et al., 2000; Quigley and Green, 1979), making these cells ideal candidates for early cell replacement strategies. Recent evidence indicates, however, that in addition to damage to

the optic nerve, prolonged elevation of intraocular pressure may also induce degeneration or loss of function of other retinal neural cell types, most notably of amacrine cells (Hernandez et al., 2009). Similar observations have been made in other retinal degenerative diseases such as retinitis pigmentosa, which is characterized not only by the loss of rod, but also of cone photoreceptors and by major morphological changes of other surviving retinal neurons (Fariss et al., 2000). Therefore early intervention may be preferable, if cell replacement strategies are to succeed, in order to restrict the number of cells types which need to be transplanted. In addition, the correct establishment of synaptic connections between transplanted RGC and native cells may be facilitated, providing that the stratified structure of the retina with its circuitry and at least some of the connections of the RGCs through the optic nerve and the optic chiasm to the lateral geniculate nucleus are preserved.
At present, research has mostly focused on the identification of suitable cells, which can be differentiated towards RGCs and their precursors, as well as the experimental conditions required for the optimal expression of their molecular markers. Furthermore, a small number of studies have investigated the electrophysiological properties of the RGC precursors generated *in vitro* and their transplantation into *in vivo* models. Although research has been conducted into the functional replacement of RGCs, and potential candidate stem cells have been identified, there are currently no cell-based therapeutic options that are either available to patients or tested in clinical trials. Establishment of cell based therapies to replace or regenerate RGCs, as with any other cell based therapy, would require validation protocols for safety, efficacy and long term survival of the transplanted cells. In the following sections we will review the potential of human ES cells, iPS cells and adult human Müller stem cells for the generation and transplantation of RGCs and their precursors.

3.1 Human embryonic stem cells as a prospective source of RGCs

Most evidence for the differentiation of ESCs into retinal progenitors and their potential for retinal transplantation has been provided by animal studies. Murine ESCs have been shown to generate RGC-like cells *in vitro* by differentiation protocols using various growth and differentiating factors. This has resulted in the expression of markers such as Ath5, Brn3b, RPF-1, Thy-1 and Isl-1 (Jagatha et al., 2009), which are characteristically expressed by RGCs. Rx/rax-expressing murine ESCs, which were treated with retinoic acid to induce neural commitment, expressed markers of RGCs and horizontal cells, displayed electrophysiological properties consistent with RGCs and were able to integrate *ex vivo* into mouse retinae (Tabata et al., 2004). Importantly, when mouse ESCs, which had been differentiated into eye-like structures, were co-cultured with retinal explants following damage to the inner retinal cells, migration into the RGC layer as well as expression of the RGC markers HuD and Brn3b were observed (Aoki et al., 2007).
Proof of concept that human ESCs can successfully differentiate into retinal neurons has been provided by xenologous transplantation. Following intravitreal injection into the adult mouse eye, human ESCs formed structures reminiscent of the developing optic cup and expressed markers of a wide range of retinal progenitors and neurons (Aoki et al., 2009).
In addition transplanted murine ESCs have been shown to integrate into the inner and outer nuclear as well as the inner plexiform layers of the retinae of host mice with retinal degeneration. Transplanted cells adopted a morphology consistent with and displayed molecular markers of a wide range of retinal neurons, such as βIII-tubulin and NeuN, calretinin, PKC-α and rhodopsin (Meyer et al., 2006).

In addition, Lamba et al. have recently provided evidence that human ESCs can generate retinal progenitors with high efficiency, expressing a number of molecular markers usually observed in the developing retina. These cells have shown exceptional correlation between their levels of expression of genes specific for differentiating neurons and the developmental stage of the retina, including markers of RGC and amacrine cells, which constitute the inner retina, i.e. HuD/C, Pax6, neurofilament-M and Tuj1 (Lamba et al., 2006).

Transplantation of human ESC-derived neural and retinal progenitors into animal models of retinal degeneration has been extensively studied by several groups. Neural precursors derived from human ESCs have been transplanted subretinally and intravitreally into mice, where they have been shown to be able to integrate into the retina and survive for long periods of time after grafting. Although these cells mostly displayed photoreceptors markers (Banin et al., 2006), such findings have provided evidence that human ESCs have the potential to form retinal neurons following engraftment. These results have been further supported by additional evidence that human ESCs can adopt a neural morphology and express neural retinal markers following transplantation and differentiating treatment in an *in vivo* murine model of RGC depletion, without giving rise to teratomas (Hara et al., 2010).

Although human ESCs have shown potential for use in RGC replacement therapies for glaucoma, major disadvantages associated with the use of human ESCs still remain. Ethical constraints relating to the use of these cells, their limited availability and safety issues regarding teratoma formation are likely to curtail the translation of ESCs for human RGC replacement to the clinical setting. Further work should therefore be aimed towards identifying alternative sources of cells that may safely and efficiently replace these cells in the glaucomatous eye without these ethical and practical constraints.

3.2 RGC differentiation of induced pluripotent stem cells

Some of the disadvantages of human ESCs have been addressed by the development of iPS cells, which have been proposed as a viable source of cells for autologous transplantation. The generation of iPS cells does not require the destruction of embryonic tissue and therefore does not have the same ethical implications as work with ESCs, which have been a limitation in a large number of developed countries. In addition, iPS cells can be derived from and tailored to the patient, making cells more widely available and rendering immunosuppressive therapy following transplantation redundant. To date, few studies have investigated the potential for iPS cells in stem cell treatment of retinal degenerative diseases, although recently some progress has been made to generate iPS cell-derived RGC-like cells.

Parameswaran et al. have recently provided evidence that iPS cells, which originated from reprogrammed mouse embryonic fibroblasts by transfection with Oct3/4, Sox2, Klf4 and c-Myc, can give rise to both RGCs and photoreceptors *in vitro*. They reported that neural induction and exposure to conditioned media from E14 rat retinal cells augmented the expression of Ath1, Brn3b, RPF1 and Irx2, which regulate RGC differentiation, while the retinal progenitor markers Sox2, Rx and Chx10 were reduced. Importantly, the same study reported that the generated RGC-like cells displayed tetrodotoxin-sensitive voltage-dependent sodium currents, which is a hallmark of functional neurons (Parameswaran et al., 2010).

Chen et al. have used a similar approach by creating iPS cells from reprogrammed murine fibroblasts, which had been transduced with Oct3/4, Sox2, c-Myc and Klf4, to generate RGC-like cells. These cells expressed markers of retinal progenitor cells, i.e. Pax6, Rx, Otx2,

Lhx2 and nestin, the levels of which were attenuated after differentiation towards a RGC fate. Differentiation was accompanied by expression of markers of RGC progenitors such as Brn3b and Isl-1, as well as Thy-1.2, a marker of mature RGCs. However, transplanted cells did not engraft into murine retina following intravitreal injection and they retained their pluripotency as demonstrated by their ability to form intraocular teratomas (Chen et al., 2010).

These studies illustrate major problems associated with the transplantation of cells derived from iPS cells, which need to be addressed. In particular, as described by Chen et al., the ability of iPS to form teratomas and therefore their potential to form cancerous growths may prove problematic. These findings suggest that preparation of iPS cell derived RGC progenitors for individual patients may need to undergo extensive validation for safety and efficacy, making them likely to be impractical and expensive for autologous therapies.

3.3 Müller stem cells as a source of RGCs for glaucoma therapies

The lack of regenerative potential of the human retina *in vivo* may be due to presently unknown inhibitory factors within the fully developed retina, since human Müller glia cells with stem cell characteristics have been reported to retain the ability to divide indefinitely *in vitro* (Limb et al., 2002). Until further research can elucidate the nature of these inhibitory factors, it is however unlikely that treatment options involving re-activation of endogenous Müller stem cells in the adult human retina can be developed. Cell replacement by transplantation of Müller stem cell-derived retinal neural progenitors may therefore currently offer a more promising strategy to restore visual function after irreversible damage or substantial loss of RGCs in glaucoma.

Müller glia with stem cell characteristics have been demonstrated to be predominantly located in the peripheral sections of the adult human retina (Bhatia et al., 2009). Human Müller stem cells can be easily isolated from cadaveric donor retina, and these cells can be grown and expanded indefinitely *in vitro,* and express markers of neural progenitor cells, such as Sox2, Notch1, Pax6, Shh and Chx10, as well as markers of Müller glia cells and retinal neurons e.g. CRALBP, HuD, PKC, and peripherin (Lawrence et al., 2007). When cultured under differentiating conditions in the presence of extracellular matrix and growth factors, enriched populations of cells expressing markers of specific retinal neurons can be obtained (Bhatia et al., 2011; Lawrence et al., 2007; Singhal et al., manuscript submitted).

This is illustrated by the fact that Müller stem cells cultured under various conditions develop a neuronal morphology and upregulate their expression of retinal neural and RGC precursor markers such as βIII-tubulin, Brn3b, Isl-1 and rhodopsin. Simultaneously expression levels of the neural progenitor marker Pax6 and the glial cell marker vimentin are also attenuated (Bhatia et al., 2011), indicating that existing Müller stem cell lines may have the potential to form RGC precursors.

However at present, intraocular transplantation studies using Müller stem cells have been conducted using mostly undifferentiated cells. Initially Lawence et al. reported integration of subretinally transplantated Müller stem cells into neonatal Lister Hooded rats and adult dystrophic RCS rats. Engrafted cells were shown to express the photoreceptor markers recoverin and rhodopsin, the RGC marker HuD as well as calretinin, which identifies RGCs and amacrine cells (Lawrence et al., 2007). Although integration of undifferentiated Müller stem cells has been observed after subretinal transplantation into adult dystrophic RCS rats, these cells were located in all retinal layers and did not selectively locate to the ganglion cell layer or adopt RGC-like morphology (Singhal et al., 2008).

Undifferentiated Müller stem cells have also been used for intravitreal and subretinal transplantation in a rat model of glaucoma. Although only few of the transplanted cells expressed the Müller glia and astrocyte marker GFAP, expression of βIII-tubulin indicates that at least some of the transplanted cells were able to adopt a neural phenotype. Interestingly, many of the grafted cells showed a migratory phenotype and aligned towards the host retina, in particular the optic nerve head, although they did not migrate and disseminate within the retina (Bull et al., 2008).
Recently it has been reported that Müller stem cells can be differentiated into RGC precursors, which integrate into the retina after intravitreal injection and can partly restore function in RGC-depleted retina as measured by electroretinography (Singhal et al., 2009). Although at present understanding of Müller stem cell differentiation towards RGC precursors is limited, previous work with this cell type has shown that they may have the potential for therapeutic regeneration of RGC function in glaucoma. In particular the maturity of Müller stem cells may potentially decrease the risk of teratoma formation. In addition, their ontogenetic proximity to retinal neurons may likely facilitate the development of protocols not only to successfully derive and transplant RGC precursors, but also to induce endogenous retinal regeneration without the need for transplantation.

3.4 Barriers to successful stem cell transplantation

Although some progress has been made regarding the successful production, delivery, integration and survival of RGC progenitors, major obstacles for successful engraftment and functional restoration remain and will be discussed below. These include the host immune response and extracellular matrix, which form a barrier for cell integration into the healthy host retina. During retinal degenerative processes, there is abnormal deposition of extracellular matrix, mainly chondroitin sulphate proteoglycans, which are responsible for the formation of glial scarring (gliosis). In addition, accumulation of microglia occurs, which has been shown to surround transplanted cells, inhibit their migration and induce their death (Singhal et al., 2008). Additionally, effective migration and integration of the transplanted cells has been suggested to be dependent upon their ontogenetic stage (MacLaren et al., 2006). These requirements will be discussed in more detail below.

3.4.1 Modulation of the host extracellular matrix

Various transplantation studies using a wide range of cells derived from ESCs as well as Müller stem cells have concluded that successful engraftment into the healthy adult retina is impeded by extracellular matrix components and the physical barrier of the inner limiting membrane (Chacko et al., 2003; Johnson et al., 2010b). This is unlikely to be influenced by the route of cell delivery, since transplantation by either intravitreal or subretinal injection did not yield integration of transplanted cells into the healthy host retina in the adult rat (Bull et al., 2008). In addition dissemination of the transplanted cells within the retina has been reported to be highly restricted (Banin et al., 2006). Conversely, integration of transplanted cells has been demonstrated in neonates (Chacko et al., 2003) or in the adult retina following injury (Chacko et al., 2003), indicating that these environments may be more permissive for successful engraftment.
Glaucomatous changes of the retina are generally accompanied by reactive gliosis as well as remodelling and deposition of extracellular matrix components (Guo et al., 2005). Increased production of chondroitin sulphate proteoglycans (CSPGs), which have been shown to

inhibit rat optic nerve regeneration after crush injury (Selles-Navarro et al., 2001) and reduce axonal and dendritic growth (Zuo et al., 1998), has been demonstrated following CNS and spinal cord damage (Bradbury et al., 2002). CSPGs have also been reported to form a barrier to cell migration following transplantation in animal models (Singhal et al., 2008) (Figure 2). Furthermore, degradation of CSPGs has been shown to enhance dendritic and axonal regeneration following brain and spinal cord injury (Bradbury et al., 2002; Zuo et al., 1998).
As a result of these findings, the effects of modulation of extracellular matrix components have recently been explored in conjunction with retinal progenitor transplantation. Evidence has been provided to show that co-administration of chondroitinase ABC or erythropoietin, which has been reported to upregulate MMP-2 (Wang et al., 2006), greatly increases the number of cells, which successfully integrate into the host retina (Singhal et al., 2008; Suzuki et al., 2007). Similarly, the integration of murine neonatal retinal cells into the adult rat host retina by *ex vivo* transplantation has been shown to be augmented by the induction of MMP-2 (Suzuki et al., 2006).

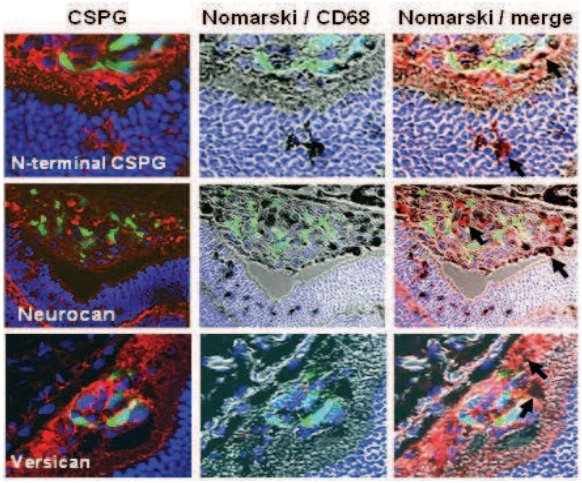

Fig. 2. Confocal imaging of rodent retina 2 weeks after subretinal transplantation of Müller stem cells. Sections on the left column shows the transplanted cells (green) surrounded by N-terminal CSPG, neurocan and versican (red). The middle column shows the same sections under Nomarski illumination to illustrate the accumulation of CD68 positive microglia (black). The column on the right shows the merged images under Nomarski illumination illustrating co-localization (arrows) of CD68 positive cells and CSPGs (red) surrounding the transplanted cells (green) (from Singhal et al., 2008).

3.4.2 Modulation of the host immune response

A successful transplantation scheme requires long term survival of the grafted cells. Allogeneic grafts induce a host immune response, leading to rejection and failure of the transplant. However cell survival is greatly increased by systemic immunosuppression of the recipient following allogeneic cell transplantation into the eye (West et al., 2010). Triple therapy with oral immunosuppressives has recently been used to increase survival of

xenografted Müller stem cells to 2 to 3 weeks, although microglia and macrophage activation was observed and transplants were destroyed after 4 weeks (Bull et al., 2008).
Activation of phagocytic microglia, the resident immune cells of the CNS, which may promote axonal degeneration of RGCs and of the optic nerve, is frequently observed during glaucoma (Ebneter et al., 2010; Yuan and Neufeld, 2001). In transplantation models, microglia prevent the migration of transplanted cells into the retina (Singhal et al., 2008) (Figure 3). Suppression of the intraocular immune response and inhibition of microglial activation by intravitreal injection of triamcinolone acetonide may therefore promote the integration and the survival of RGC precursors into retinae with glaucomatous changes. Intravitreal injection of triamcinolone acetonide in combination with oral immunosuppression and anti-inflammatory medication has previously been shown to greatly reduce microglial activation against the xenograft (Singhal et al., 2008; Singhal et al., 2010).

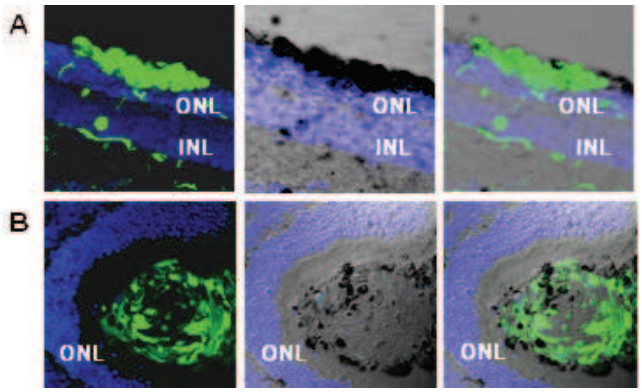

Fig. 3. **A**. Müller stem cells (green) accumulate in the subretinal space and do not migrate into the retina. Middle image shows Nomarski illumination identifying CD68 positive cells (black) in the same retinal section. Right figure shows Nomarski illumination identifying co-localization of transplanted cells with microglial cells expressing CD68 (black).
B. Transplanted cells can be seen forming a large cluster in the subretinal space 2 weeks after transplantation. Middle image shows localization of microglia (black) around the transplanted cells (green). Right figure shows microglia (black) surrounding the transplanted cells (green) and resembling a granuloma-type structure (From Singhal et al., 2008).

In experimental animal models, immune-tolerization of embryos or neonates by intraperitoneal injection of grafted cells may be used to further reduce the host immune response (Billingham et al., 1953).

3.4.3 Ontogenetic stage of transplanted cells

Currently the role played by the ontogenetic stage of transplanted RGC precursors upon their integration into host retina, as well as on functionality of the engrafted cells, has not been investigated. Previous transplantation studies have demonstrated that stem cells isolated from adult individuals rarely migrated into the healthy adult retina (Johnson et al., 2010a; Lawrence et al., 2007; Singhal et al., 2008), while embryonic and neonatal retinal

progenitors and other stem cells have been shown to successfully integrate into the host retina (Warfvinge et al., 2001; Wojciechowski et al., 2004), suggesting that the developmental stage of the transplant may be crucial for successful migration and functional integration.
Several studies have investigated the role of the developmental stage of grafted retinal neurons for successful incorporation into the host retina. Based on this work, it has been concluded at least in the case of photoreceptor transplantation that early postnatal post-mitotic precursors or cells of a similar ontogenetic stage are the most promising candidates for transplantation in terms of their ability to migrate and disseminate into the retina and differentiate towards a functional phenotype (MacLaren et al., 2006).
However, more recent studies have suggested that there may be no need for transplantation of photoreceptor progenitors for these cells to integrate, as fully mature photoreceptors retain the ability to integrate into the mature retina upon transplantation (Gust and Reh, 2011). Moreover, integration of photoreceptors derived from human Müller glia into the degenerated rat retina has shown to be independent from NRL expression by these cells (Jayaram et al., unpublished observations).
In addition the developmental phase of transplanted cells will likely have major implications on treatment safety, with less differentiated cells posing a greater risk of tumorigenesis. In fact, a number of studies using cells derived from embryonic stem or iPS cells have reported the occurrence of teratomas (Arnhold et al., 2004; Chen et al., 2010), whereas no formation of cancerous growths was reported after transplantation of adult-derived stem cells.
In summary, future research will be needed to elucidate the effects of the ontogenetic stage of transplanted RGC precursors on graft integration, function and safety.

3.5 Strategies to measure functional outcome

With the development of methods for the transplantation of RGC in glaucoma, the measurement of functional outcomes will become increasingly important. It can be anticipated, however, that these will encompass techniques currently available for the monitoring of disease progression. Electrophysiological measurements are widely used to assess glaucomatous damage both in patients and in experimental animal models and will likely continue to play a major role in evaluating treatment success. Some of these protocols have been standardized by the International Society for Clinical Electrophysiology of Vision (ISCEV) guidelines (Holder et al., 2007; Marmor et al., 2009), although they may be complemented by other methods established for laboratory use.
The pattern ERG is currently one of the most useful techniques to assess glaucomatous damage in patients. It generally utilizes a black and white checkerboard stimulus with pattern reversal as prescribed by the ISCEV standards (Holder et al., 2007). The pattern ERG has been shown to be reduced in patients with glaucoma and correlates with visual field defects (Wanger and Persson, 1983). In addition the use of variable check sizes may be advisable to assess the extent of glaucomatous change (Bach et al., 1988). Recently multifocal pattern electroretinograms have been demonstrated to be reduced in glaucoma patients (Monteiro et al., 2011; Stiefelmeyer et al., 2004), although other studies have reported that localized reductions of the signal amplitude could not be correlated with visual field defects (Klistorner et al., 2000). Since this method requires good accommodation and fixation (Holder et al., 2007), it is widely used in human subjects, while its applicability to animal models is limited.
Preclinical studies will likely favour methods employing Ganzfeld stimulation, which are relatively easy to apply to a laboratory setting. The most commonly used of these is the

scotopic threshold response, a low intensity light response with stimulation below the psychophysical threshold, which has be ascribed to RGC function, although it may species-dependently contain contributions from amacrine cells (Frishman et al., 1996; Korth et al., 1994; Sieving, 1991). More recently the photopic negative response has been established as a measure of RGC function (Viswanathan et al., 1999), although other cellular origins, such as glia and amacrine cells, have been suggested (Machida et al., 2008). However, at present this has not been assimilated into ISCEV guidelines.

Pattern reversal, pattern onset/offset or flash visual evoked potentials can be used to assess RGC and optic nerve function. Although they are usually employed in a clinical setting (Odom et al., 2010), especially the flash, the pattern onset/offset visual evoked potentials may potentially be used for experimental applications in animals (Huang et al., 2011; Ver Hoeve et al., 1999). Recently multifocal mapping of visual evoked potentials has been developed (Hasegawa and Abe, 2001), but has not been widely applied to practice. However, for clinical purposes perimetry will remain important, as gains in the visual field of patients may indicate whether potential RGC cell therapies are successful.

4. Optic nerve regeneration

It has been considered critical for the functional success of RGC replacement therapies in glaucoma that transplanted cells form axons, restore the optic nerve and establish new connections with their physiological targets. The optic nerve has traditionally been thought to be incapable of renewal, with axonal damage invariably leading to the degeneration of RGC somata and resulting in the irreparable loss of vision.

A range of studies has investigated the effects of peripheral nerve transplantation on RGC survival as well as axonal sprouting and re-growth. Extensive evidence has been presented that autologous grafts of peripheral nerves can protect axotomized RGCs from cell death and in addition can promote the regeneration and re-growth of axons. Some studies have even shown that after transplantation of peripheral nerves, RGCs regenerated long axons, which extended into the superior colliculus, where they formed synapses in their physiological target region (Aguayo et al., 1991; Vidal-Sanz et al., 1991).

Other cell types such as OECs and macrophages have been suggested to augment axon formation. The promoting effect of OECs on neurite formation may likely be contact-mediated (Leaver et al., 2006). In addition, macrophages have been reported to promote axonal growth, probable through the release of oncomodulin and activation of the protein kinase Mst3b and Ca^{2+}/calmodulin kinases as downstream effectors (Lorber et al., 2009 ; Yin et al., 2006).

Distinct growth factors have been identified which may affect optic nerve regeneration. A combination of fibroblast growth factor 2 (FGF2), neurotrophin 3 (NT3) and brain derived neurotrophic factor (BDNF) (Logan et al., 2006) has been reported to stimulate axonal outgrowth of RGCs. Furthermore a range of molecules have been identified, which can reduce dendrite formation, e.g. Nogo-A, myelin-associated glycoprotein and components of the extracellular matrix such as proteoglycans (Koprivica et al., 2005; Su et al., 2009; Wong et al., 2003). Many of these inhibitory factors converge on the small G-protein RhoA, inhibition of which has been shown to result in stimulation of axon formation (Bertrand et al., 2005).

Interestingly, the length of new axons grown from cultured RGCs has been reported to be reduced after the developmental age at which synaptic connections in the superior

colliculus are formed, although the proportion of cells generating axons was not altered (Goldberg et al., 2002).
The formation and guidance of axons from RGCs to their targets during development have been intensively investigated. Netrins, semaphorins, laminin, erythropoietin-producing hepatocellular receptor/Eph receptor-interacting protein, Wnt and slits have been shown to act as chemo-attractants and repellants during optic nerve development in the embryo (Erskine and Herrera, 2007; McLaughlin and O'Leary, 2005). Interestingly, some of these guiding signals have been reported to be retained or restored following injury in the adult brain (Bahr and Wizenmann, 1996), which may help to guide axons formed by transplanted RGCs to the right targets. Additionally it has been shown that following transplantation of embryonic retinal tissue, connections to the superior colliculus are successfully established (Seiler et al., 2010).

5. Stem cell mediated neuroprotection in glaucoma

The pathophysiological mechanisms implicated in RGC loss seen in glaucoma have led to the development of neuroprotective strategies becoming a major focus of current glaucoma research (Danesh-Meyer, 2011). Contemporary research in stem cell mediated neuroprotection for glaucoma has been developed on the backdrop of promising work performed in models of neurodegenerative disease affecting other parts of the CNS.
Glaucomatous RGC loss and neuronal degeneration in other neurodegenerative conditions share mechanisms such as oxidative stress, impairment of axonal transport, excitotoxicity and inflammation (Baltmr et al., 2010) making neuroprotective strategies relevant to patients affected by both conditions.
Stem cell derived strategies for neuroprotection, if successful, offer several theoretical advantages over conventional pharmacological approaches. Should transplanted cells integrate within the host retina, it is possible that a single treatment may provide long term neuroprotection offering support to surviving neurons. The observation that endogenous neural stem cells are able to migrate to the site of injury in ischaemic stroke and differentiate into mature neurons (Felling and Levison, 2003), gives rise to the possibility of a similar phenomenon occurring with transplanted cells in the context of glaucoma, with such cells potentially responsible for the provision of local support.
Stem cells are able to facilitate local neuronal survival by the production of several neurotrophic factors. This multifactorial effect has been demonstrated in animal models of CNS disease (Corti et al., 2007) and work in a rodent model of Parkinson's Disease showed that neural stem cell transplantation conferred a more significant neuroprotective benefit than both a single injection of neurotrophins or prolonged delivery via local infusion (Yasuhara et al., 2006). Transplantation of neural progenitors in animal models of neurodegenerative disease has been shown to confer neuroprotection via an immunomodulatory mechanism (Pluchino et al., 2005). Alteration of the microenvironment surrounding damaged RGCs, perhaps through immune mediated actions of transplanted cells, may help promote local neuronal survival.
A major beacon of hope in stem cell research is the concept of autologous transplantation. Such a strategy would minimise the risk of graft rejection and prevent a lifetime of potentially toxic immunosuppressive therapy for patients. Neuroprotective strategies involving Müller stem cells, bone marrow derived mesenchymal stem cells and OECs offer realistic potential for autologous transplantation. However, more work is still necessary to

design practical approaches to obtain suitable tissue for this purpose, as well as to derive functional cells that can be used for transplantation. Should current concerns regarding the safety of iPS cells for therapeutic use be overcome (Jalving and Shepers, 2009), these reprogrammed adult somatic cells offer an exciting avenue for the development of autologous therapies in the future.

Mesenchymal stem cell mediated neuroprotection has been demonstrated following transplantation in various models of retinal degeneration (Arnhold et al., 2007; Inoue et al., 2007; Lu et al., 2010; Zhang and Wang, 2010). This phenomenon is likely to be secondary to the secretion of neurotrophic factors such as BDNF, ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), insulin like growth factor 1 (IGF1) and FGF2 (Cho et al., 2005; Labouyrie et al., 1999) which are known to offer protection to damaged retina. These observations, coupled with promising results showing neuroprotection in models of CNS degenerative disease (Andrews et al., 2008; Karussis et al., 2008; Parr et al., 2007; Torrente and Polli, 2008), have led to this category of stem cells becoming a focus for the development of cell-based neuroprotective strategies to treat glaucoma.

Disruption of the retrograde axonal transport of BDNF has been shown to be involved in the pathophysiology of glaucoma (Pease et al., 2000) and attempts to upregulate expression of BDNF (Martin et al., 2003) and CNTF (Pease et al., 2009) using gene therapy have been shown to attenuate RGC loss in experimental models. A reduction in RGC loss has been observed in rodents with raised intraocular pressure following intravitreal transplantation of mesenchymal stem cells (Johnson et al., 2010a; Yu et al., 2006). The latter reported increased levels of CNTF, BDNF and FGF within the retinae of treated eyes, which were hypothesised to be responsible for this neuroprotective effect. Survival of the cells was observed at up to five weeks, but currently there is a lack of data describing long term graft survival and a prolonged neuroprotective effect, both of which will be essential for such a therapy to be translated to the clinic.

Despite suggestions that mesenchymal stem cells may possess a capacity to migrate from the systemic circulation into diseased tissue, migration into chronically damaged neural tissue is regarded as being limited, and hence strategies for cell delivery would be best served by direct injection into affected tissue. In the context of glaucoma models, cells administered via an intravenous approach were unable to be detected in the eye and had no effect in attenuating RGC loss (Johnson et al., 2010a).

The neuroprotective effect of transplanted cells may be optimised further by enhancing the neurotrophin secreting ability of cells through either cytokine driven protocols or gene therapy techniques. Proof of concept for this idea was demonstrated in a model of cerebral ischaemia where intravenous infusion of mesenchymal stem cells genetically modified to deliver BDNF to the cerebral circulation provided a greater neuroprotective effect than untreated cells (Nomura et al., 2005). This principle has been successfully applied to a rodent model of RGC damage induced by optic nerve transection (Levkovitch-Verbin et al., 2010). Mesenchymal stem cells were induced to secrete high levels of BDNF, VEGF and Glial Derived Neurotrophic Factor by using a cytokine driven protocol *in vitro*. Intravitreal transplantation of both modified and untreated mesenchymal stem cells produced similar neuroprotective effects when compared to sham injection. One interpretation of these findings would be that even small amounts of trophic factor release, as seen with untreated cells, may confer neuroprotection. However a more realistic argument may be that the severity of optic nerve transection is such that even the higher levels of trophic factors delivered by the modified cells would be unlikely to prevent RGC death. Further research

into the role of cell populations that have enhanced neurotrophin secreting capability in models of glaucoma may provide further insight into the therapeutic potential of such an approach.

Inflammation has frequently been associated with neurodegenerative disease. It is commonly observed as a consequence of acute injuries including trauma and stroke, but is also a characteristic feature of demyelinating disease where autoimmune processes are central to the pathophysiology. Mesenchymal stem cells derived from the bone marrow are known to have the ability to modulate the inflammatory response. There is much hope and optimism in the field of multiple sclerosis that these cells may provide *in situ* immunomodulation and neuroprotection (Payne et al., 2011) with the results of clinical trials eagerly awaited. It is quite feasible that this mechanism may be applicable to glaucomatous RGC loss, however further studies are required to investigate this possibility.

The observation that OPCs exhibit neuroprotective properties *in vitro* (Wilkins et al., 2001) has led to some interest in their role as a potential candidate for cell-based therapies in a model of glaucoma. Interestingly OPCs were only able to demonstrate a neuroprotective effect following concomitant activation of pro-inflammatory cells using zymozan (Bull et al., 2009). The neuroprotective effect was not contact-mediated and was attributed to the release of diffusible trophic factors from the activated OPCs. A potential risk of transplanting such cells into glaucomatous eyes is the potential of excessive myelination, which carries the theoretical risk of blocking the transmission of light within the eye and reducing the electrical conduction of RGCs. However further studies into the nature of the trophic factors released by these cells may aid the design of further novel neuroprotective strategies to treat glaucoma.

OEC transplantation has been observed to increase axonal regeneration in models of spinal cord injury (Ramon-Cueto and Valverde, 1995). These initial observations led to the development of further studies into the potential of these cells to develop novel treatments for optic nerve disorders and glaucoma. *In vitro* work has demonstrated that OECs cause ensheathment of RGCs without the process of myelination occurring (Plant et al., 2010). Transplantation of OECs into the distal stump of transected optic nerves provided further evidence of regeneration of several axons (Li et al., 2003; Wu et al., 2010) that were supported by the transplanted cells. Following transretinal delivery into normal rodent eyes, OECs migrate along the RGC layer into the optic nervehead demonstrating ensheathment of RGC axons by the cytoplasm of transplanted cells (Li et al., 2008). It is possible that this process may provide some mechanical support to compromised axons, which may subsequently be able to maintain sufficient functional vision if therapies can be developed for patients with end stage glaucoma.

Evidence from models of spinal cord transection suggests that OEC transplantation is associated with an increased secretion of neurotrophins such as BDNF which appears to correlate with the neuroprotective effect (Sasaki et al., 2006). However it was not clear whether the BDNF was secreted by the transplanted cells or by activation of endogenous cells. Attempts to combine OEC transplantation with concomitant neurotrophin administration have shown promising results to date. Combination therapy in a model of optic nerve crush resulted in restoration of the latency of the visual evoked potential to almost 90% of normal levels with retrograde RGC labelling suggesting of axonal regeneration (Liu et al., 2010).

Future studies using these cells directed towards attenuating glaucomatous RGC loss may focus upon the potential of external support of RGC axons exiting via the lamina cribrosa as

well as internal neuroprotection mediated by the provision of trophic factors. In addition further study into the functional characteristics of OECs is required as well as investigation of the effects of OEC in models of experimental glaucoma.

The perfect stem cell-based therapy to treat glaucoma would involve the activation of endogenous stem cells to repair damaged RGCs and thus restore function. The damaged CNS lacks plasticity and neuronal regeneration is notoriously difficult due to a lack of trophic cues (Hou et al., 2008) and the inhibitory nature of the microenvironment (Asher et al., 2001). Nevertheless there is growing evidence that endogenous neural stem cells may proliferate in response to brain injury such as stroke (Felling and Levison, 2003). Although only a proportion of new cells differentiate into new neurons and survive in the long term (Naylor et al., 2005), methods have been established to enhance the proliferation of endogenous neural stem cells following ischaemic injury (Ninomiya et al., 2006).

With respect to damaged neurons within the retina, it may be the Müller glia that hold the key for endogenous reactivation. Their well-documented capacity to regenerate retinal neurons in the teleost retina (Bernardos et al., 2007; Fimbel et al., 2007) and the known presence of similar cells in adult human retina (Lawrence et al., 2007) would make these cells a promising target around which studies of endogenous stem cell repair could be developed.

6. Conclusion

The rapidly evolving field of stem cell research offers exciting potential in the long term for innovative therapies moving from bench to bedside in patients who are affected by advanced glaucoma. Although regeneration of the optic nerve itself may be unrealistic with current scientific knowledge, further studies into local retinal ganglion replacement and neuroprotective mechanisms using transplanted stem cells may offer hope that such treatments may be translated to patients in years to come.

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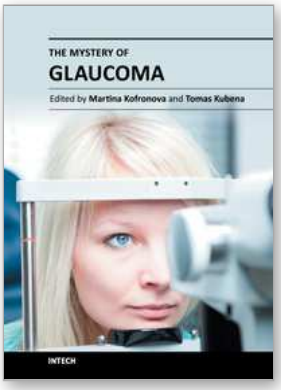

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Since long ago scientists have been trying hard to show up the core of glaucoma. To its understanding we needed to penetrate gradually to its molecular level. The newest pieces of knowledge about the molecular biology of glaucoma are presented in the first section. The second section deals with the clinical problems of glaucoma. Ophthalmologists and other medical staff may find here more important understandings for doing their work. What would our investigation be for, if not owing to the people's benefit? The third section is full of new perspectives on glaucoma. After all, everybody believes and relies – more or less – on bits of hopes of a better future. Just let us engage in the mystery of glaucoma, to learn how to cure it even to prevent suffering from it. Each information in this book is an item of great importance as a precious stone behind which genuine, through and honest piece of work should be observed.

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