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STEM CELL THERAPIES FOR RETINAL REPAIR AND REGENERATION

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Abstract: Neural cell damage is the main feature of retina degenerative disorders and constitutes the major cause of blindness in patients affected by retinal disease. Present treatments aim to prevent disease progression but do not reverse lost vision, for which stem cell-based therapies are the only hope for restoration or maintenance of visual function in patients affected by severe disease. This review summarizes recent progress in the stem cell field and describes advances made on the clinical application of these cells for treatment of retinal degenerative diseases. It also describes recent research in the field that is being actively pursued to promote endogenous regeneration of the neural retina.

Keywords: retina, embryonic stem cells, induced pluripotent stem cells, tissue specific stem cells, Müller cells; retinal ganglion cells; retinal degeneration; transplantation; neurotrophins.

1. Introduction

New developments in the stem cell field have broadened the prospects of designing cell-based therapies to repair and restore visual function. Epidemiological studies on diseases such as age-related macular degeneration (AMD) (1), glaucoma (2) and diabetic retinopathy (3), suggest that blindness and severe visual impairment caused by these conditions are on the rise, therefore creating significant economic burden to health care systems in the world. Current treatments can control disease progression but cannot restore lost function, for which new therapies are needed to repair the retina and restore visual function. Research undertaken in the stem cell field in recent years has raised the hope for the use of these cells as therapeutic agents to prevent blindness and restore visual.

Most studies in the retina regenerative field have been aimed at replacing neurons by stem cell transplantation. However, neural replacement by cell grafting has not been successfully achieved as this process requires integration of transplanted cells within major neural networks. Nonetheless, partial restoration of retinal function has been observed after experimental stem cell grafting, suggesting that stem cells may constitute an important source of neuroprotective factors to repair neural damage and this is a subject of current investigations in the field (4-6). Furthermore, following the discovery of the presence of Muller glia with stem cell characteristics in the adult human retina (7, 8), approaches to induce self-repair (endogenous regeneration) are at present being investigated by several groups. Current knowledge in the transplantation and endogenous regeneration research fields are discussed in the following sections.

1.2 Retina degeneration as a cause of visual impairment and blindness

Retina degenerative diseases are characterized by neural cell damage, which is the ultimately cause of visual impairment and blindness. The retina harbours six different types of neurons, namely cone and rod photoreceptors, bipolar, horizontal and amacrine cells, and retinal ganglion cells (Fig 1). Two macroglia populations, comprising Müller glia and astrocytes are also present in the retina, with Müller cells providing structural and metabolic support to all retinal neurons. Müller glia and retinal neurons have a common progenitor (9) but the origin of retinal astrocytes has been debatable. Evidence has been presented that astrocyte migration along the already formed optic nerve appears to be dependent on the presence of vascular progenitors (10).

During the visual process, rods and cones, the light sensitive neurons, respond to light photons by producing neural impulses that trigger a cascade of neuronal processes. This leads to retinal ganglion cells synapsing with their targets in the brain to allow visual perception. Adjacent to the neural retina is the retinal pigment epithelium (RPE), which forms the blood retinal barrier (11) and plays a very important role in the visual cycle and metabolic functions of retinal neurons (12). When damage occurs to the RPE or selective populations of retinal neurons, a reorganization of the neural circuit ensues. This results in death of most neural cell populations in the retina, causing loss of visual function and consequently blindness. These pathological features are clearly illustrated during the development and progression of prevalent retinal degenerative diseases. For example, during age related macular degeneration (AMD), initial damage is observed in the RPE, but during later stages photoreceptor cell death occurs due to impairment of their metabolic protection by RPE cells (13, 14). In other conditions, such as retinitis pigmentosa, degeneration of photoreceptors leads to

retinal ganglion cell (RGC) loss due to axon compression and neural reorganization(15), whilst damage to the optic nerve and RGC in glaucoma leads to photoreceptor and horizontal cell damage with consequent blindness (16).

Due to the complex network of the neural retina, methods to promote retina regeneration by stem cell transplantation are exceptionally challenging. During early retinal disease, regeneration of a single neural cell type may prove more feasible than in late disease when all neural cell types have been compromised and spread neural reorganization has occurred. Any approach to regenerate the retina will require not only 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 (17, 18).

2. Stem cell potential for retina regenerative therapies

Regenerative medicine promises vast benefits for patients with progressive retinal disease, and stem cells provide an unlimited source of different cell types for potential therapies, as well as for drug screening and new drug development. Progress in the isolation and propagation of stem cells from various sources has opened the possibility of generating all types of retinal cells for therapeutic application. It is however possible that stem cells obtained from different sources may differ in their ability to repair or restore retina function upon transplantation into the eye, and understanding the potential of various stem cell sources for their clinical application may aid in the development of effective retinal therapies.

Pluripotent stem cells, including human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) have been widely used to investigate the ability of these cells to repair or regenerate the retina in animal models of retina degeneration (19-21). Other stem cells being explored for their potential to regenerate the retina include adult stem cells such as human Müller stem cells (22-24), retinal progenitors (25), which are closely related to Müller glia (26), and mesenchymal stem cells derived from the umbilical cord and bone marrow (27, 28). Details on the use of these cell types in experimental studies to regenerate the retina will be described in the following section.

2.1 Embryonic Stem Cells

Embryonic stem cells (ESC) are derived from the inner cell mass of the blastocyst (29) and are capable of differentiating into most retinal cell types under specialized culture conditions *in vitro.* Because of their ability to self-renew and to differentiate into multiple neurons and glia (30), ESC have been widely recognized for their potential to developing retinal cell therapies. Efficient differentiation of ESC into retinal progenitors has been obtained by inhibiting the Wnt and BMP pathways combined with the addition of IGF-1 or a combination of Nodal and Wnt antagonists (30). In addition, defined protocols to obtain differentiated RPE cells from human ESC for clinical application have been successfully established by various investigators (31, 32). These protocols have been applied to the preparation of RPE cells that have been used in initial clinical trials to treat patients affected by Stargardt's macular dystrophy and age related macular degeneration (AMD) (33, 34). Transplantation of retinal neurons derived from hESC has been widely explored in rodent

models of retina degeneration (35-37) and normal non-human primate retina (38), but clinical application of these cells has not yet been achieved.

2.2 Induced Pluripotent Stem Cells (iPSC)

Pluripotency of adult somatic cells such as keratinocytes (39), hematopoietic stem cells (40) and dermal fibroblasts (41) can be achieved by transfection with four transcription factors associated with the embryonic stem cell state: Oct4, Sox2, cMyc, and Klf4 (42). However, because of the tumorigenic potential of c-Myc and the pathogenic risk of using viral vectors used to generate iPSC, various modified protocols have been designed to address these safety issues (42, 43). Despite being derived from adult tissue cells, these pluripotent cells share many features of ESC, including DNA methylation, gene expression and chromatin state (44). A major advantage of iPSC over ESC is their potential for autologous use, which could avoid the need for immunosuppressive therapies upon transplantation. Although these properties make these cells more desirable for regenerative medicine, there is evidence that iPSC lines can harbor different genetic and epigenetic signatures (45). On this basis, extensive investigations to derive iPSC lines with stable genomic architecture are being undertaken for application into the clinic.

2.3 Adult Stem Cells

Ethical and tumorigenic concerns on the use of ESC and iPSC in cell therapies and limited methodology available to differentiate these cells into mature neural cell types, have driven investigators to explore the potential application of adult stem cells in retinal regenerative medicine. Mesenchymal stem cells derived from bone marrow and umbilical cord blood (46-48), adipose tissue (49), as well as Müller glia and progenitor cells (22-25) have been induced to differentiate *in vitro* into retinal neurons and have been transplanted into the retina. RPE cells have been reported to trans-differentiate towards retinal neurons (50), but convincing evidence that these cells have the potential to replace retinal neurons or RPE cells has not yet been presented. Adult neural stem cells derived from the adult hippocampus have also been transplanted into the eyes of experimental models of retinal disease with various outcomes (51, 52), and despite lack of evidence that mesenchymal or neural stem cells can regenerate the retina, these cells have been shown to be a potent source of trophic factors that aid in neural cell protection and recovery (46, 53). On this basis, their potential use may be likely to be restricted to cell-based neuroprotective strategies rather than neuronal replacement.

Retinal specific stem cells, known as Müller glia, have been identified in the neural retina of the human eye (7, 54), and they have been investigated for their potential use in retina regenerative therapies. Müller glial cells constitute the major glial cells of the neural retina. They expand across the whole width of the retina and provide structural and metabolic support to retinal neurons (55, 56). Müller glia are responsible for the spontaneous regeneration of the zebrafish observed throughout life (57). These cells have been found in all vertebrate species including the chick (58) and small rodents such as mice (59) and rats (60), but their endogenous regenerative ability is severely limited in chicks and small mammals (61). Although Müller glial cells with stem cell characteristics are present in the adult human retina (7), there is no evidence for their endogenous regenerative ability. However, human Müller stem cells can be cultured indefinitely *in vitro* and display features of pluripotent progenitor cells in that they have the potential of unlimited cell

renewal, express markers of stem cell and neural precursors and differentiate into cells with characteristic markers and functions of retinal neurons *in vitro* (7, 23, 24, 62). Experimental transplantation studies have shown that enriched populations of neural retinal precursors derived from Müller stem cells significantly improve visual function in animal models of retinal ganglion cell depletion (22, 24) and photoreceptor degeneration (23).

3. Neural stem cells during retinal development and adult life

3.1 Embryonic development of the retina

During embryonic development, eye formation begins with evagination of the neural tube on either side of the developing forebrain. This leads to the formation of optic vesicles that further fold to form a double layered optic cup. Further invagination of the optic cup drives differentiation of the optic stalk, neural retina and retinal pigment epithelium(63). Cell lineage studies using tracers or retroviruses have shown that all neural retinal cells derive from a common multipotent retinal progenitor cell (RPC)(9, 64). Current studies suggest that here is an order to which the retinal neurons and glia are generated, however they do overlap with each other. Retinal ganglion cells are the first retinal neurons to develop, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar and finally Müller glial cells (64). Several models have been hypothesised to describe cell fate specification in the retina. Progenitor cell division can therefore be asymmetric, symmetric mitotic or symmetric post mitotic during retinal cell differentiation. The 'competence model' of retinal cell fate determination suggests that each RPC goes through different states driven by extrinsic cues (65). During these 'competence' states, the progenitor cells can form only a particular subtype of retinal cell, but also have the ability to switch states at any given time. Another model suggests that early transcription factor expression in RPCs determines the production of future retinal cell types (66). It has been also suggested that all RPCs are comparable and extrinsic factors drive retinal fate specification (67). It is therefore likely that a combination of these models may drive retinal cell fate determination, and this is the subject of investigations in the retinal development field.

Retinal progenitor cells are characterised by their expression of multiple transcription factors that drive their differentiation into mature retinal neurons through a sequence of activation driven by local and environmental signals (68, 69). Through activation of the downstream basic helix-loop-helix (bHLH) transcription factors Hes1 and Hes5, Notch pathway activation maintains the progenitor cell state. This prevents differentiation and promotes rapid proliferation which is thought to be required to complete the formation of all the retinal cell types (70, 71). To drive neural differentiation, Notch signalling is downregulated causing increased expression of bHLH (Mash1 / Math5 / Math3 / NeuroD / Ascl1) and homeodomain (Chx10 / Pax6 / Six3 / Crx /Otx2 /Prox1) activators (72, 73). Müller glia formation however, requires continual activation of Hes1 and Hes5 in the RPCs (74, 75), which is associated with the progenitor-like roles ascribed to Müller glia. Retinal ganglion cell development is driven by sonic hedgehog (Shh) signalling in a positive feedback loop, where already formed RGCs secrete Shh to promote further differentiation. RGC differentiation is accompanied by the expression of Atoh7, Pou4f1/2/3 (previously Brn3), Isl 1 and NeuroD1, concomitant with a continued expression of the progenitor markers Pax6 and Math5 (70, 76). Horizontal cells are generated by co-

expression of Math3 and Pax6 , and amacrine cells also express these factors in addition to NeuroD . Photoreceptor generation is characterised by expression of the transcription factors Otx2 and Crx, where NR2E3 is specific to rod cell fate, and thyroid hormone receptor β2 (TRβ2) determines the type of cones generated (77, 78). Although many studies have investigated the control of retinal cell fate determination, there is not a complete understanding of the interactions between intrinsic and extrinsic cues that direct neural differentiation in the human retina. It is therefore important for future studies to build upon the regulation of these factors and mechanisms involved, and new in vitro modelling of retinal organoid formation by ESC and iPSC may further confirm these mechanisms across different species.

3.2 Retinal organoids formed by ESC and iPSC in vitro

Recent advances in stem cell research have shown that through targeted differentiation, human iPSC and ESC can mimic early retinal development (79-81). There has been much progress in the growth of 3D models of retina *in vitro* which has not only facilitated investigations on the mechanisms of retinal neuron specification (79, 82, 83) and retinal lamination (84), but also on the testing of new drugs for potential use in retinal therapies. Retinal organoid formation *in vitro* allows mapping of the molecular timings associated with retinal neuron development that are difficult to observe *in vivo*. Investigations in the field have shown that both mouse and human ESC are able to form 3D optic cup-like structures following early inhibition of the Wnt signalling pathway, addition of matrigel, sonic hedgehog agonists and supplementation with foetal bovine serum (FBS) (79-81). Eiraku et al have described four phases during retinal organoid development of mouse ESC that mimic retinal organogenesis *in vivo*. In the first stage, a spherical vesicle emerges from the embryoid body, which flattens in the second stage. During the third stage, the junction between neural retina, RPE and embryoid body narrows before invagination of the neural retina on the final stage (83). The process of optic cup formation by human ESC occurs at approximately 24 days from the start of the differentiation process (79). This also coincides with the expression of Pax6 and Brn3 positive cells which are early markers of retinal ganglion cell differentiation (RGC). Lineage studies using a *venus* cDNA knock in gene for the photoreceptor marker Crx have shown that photoreceptors start emerging at days 28-34 and their number gradually increase over time, expressing recoverin at day 60 and rhodopsin and Nrl by day 126 after organoid initiation (79, 83, 85). There is limited knowledge of the development of other neural retinal cells including amacrine, bi-polar and horizontal cells, however markers such as ap2α (amacrine) Ptf1a, calretinin (horizontal/amacrine) and PCK, VSX2 (bi-polar) positive cells have been observed in retinal organoids following seven to eighteen weeks after initiation of organoid formation (81, 85). It has been generally accepted that Müller glia are the last retinal cells to form during retina development (9), and in human ESC derived retinal organoids, Müller glia staining for glutamine synthetase has been observed in 90 day old organoids (85). Genes associated with mature Müller glia markers, including DBI, GNAI2, GLUL, and DKK3 have been also observed at 37 days following induction of organoid formation by human ESC (85), but it is not clear whether these are restricted to Müller glia or other cells present in the retinal organoids. A diagram illustrating the various stages observed during the development of retinal organoids from ESC and iPSC *in vitro* is shown in Fig 2.

Another important question in biological studies of optic cup formation *in vitro*, is whether retinal organoids formed in the laboratory are able to exhibit retinal function. Using patch clamping, Zhong et al have shown that organoids derived from hiPSC not only contain photoreceptors with outer-

segment-discs at 27 weeks after initiation of organoid formation, but that they respond to light stimulation in a comparable manner to rod photoreceptor cells *in vivo* (86). Other groups have modified the original protocols developed by Nakano et al (79) to encourage the development of organoids which favour the production of particular retinal cell types, such as photoreceptors (4), or retinal ganglion cells (87) which may have potential uses for retinal cell transplantation and drug screening.

4. Prospects for retinal repair and regeneration using stem cells

4.1. ***Stem cell transplantation***

Knowledge gained in the stem cell field during recent years has widened the prospects of developing cell-based therapies to repair the retina that has been irreversibly damaged by disease or injury. Stem cell transplantation has been extensively performed in several experimental models of retinal degeneration, but cell therapies to regenerate the human neural retina are far from being implemented in the clinic, and still constitute a major challenge. Understandably, the majority of retinal transplantation studies published to date have been addressed to regenerate the retina in animal models of photoreceptor degeneration. This is possibly due to the fact that photoreceptor damage is the main feature of retinal degenerative diseases that prevail in large populations, and that include retinitis pigmentosa and age related macular degeneration. Cells used for transplantation in animal models of photoreceptor degeneration have involved, amongst others, Schwann cells(88), brain-derived precursor cells (89, 90), photoreceptor precursors derived from the immature retina *in vivo* (91), or from pluripotent stem cells (4), ESC derived neural precursors (92), mesenchymal stem cells (93) and Müller stem cells (23). Successful outcomes in models with photoreceptor degeneration with partial restoration of visual function have been reported following photoreceptor cell transplantation in the subretinal space, and these effects were originally attributed to anatomical integration of the grafted cells into the neural circuitry (91, 94, 95). However, recent observations from various groups have shown that transplanted photoreceptor cells do not integrate into the retina but that they transfer intracellular material to the host cells. As a result, fluorescent proteins used to trace transplanted cells are transferred into adjacent host retinal cells, giving the appearance of transplant integration (96-98). These studies have led investigators to reconsider whether improved visual function by cell transplantation should be aimed to replace neurons or to deliver neuroprotective factors to repair the degenerated retina. Other factors limiting the success of stem cell transplantation into the degenerated retina constitute the glia scarring and microglia accumulation, often occurring as a result of degenerative disease (18, 99). Addressing these problems may facilitate the migration and survival of transplanted stem cells.

In contrast with the high number of transplantation studies aimed at restoring photoreceptor cell function, stem cell studies to repair or regenerate the retinal ganglion cell layer and optic nerve have been limited. Retinal ganglion cell (RGC) degeneration occurs in conditions such as glaucoma, ischaemic optic neuropathies and inherited optic neuropathies, which also constitute major causes of blindness and for which stem cell based therapies, may be beneficial. Various sources of stem cells have been investigated for their ability to repair RGC function in animal models of glaucoma-like disease and include bone marrow mesenchymal stem cells (100), oligodendrocyte precursors (101), iPSC derived trabecular meshwork cells (102), olfactory ensheathing cells (103) and Müller stem cells (22, 24). As seen with transplantation involving photoreceptors, stem cell studies to repair

or regenerate the glaucomatous-like retina have proved to have a beneficial effect as demonstrated by improvement in RGC function. However, evidence for transplant cell integration with axon extension to the optic nerve and brain has not been achieved, for which it has been suggested that neurotrophic and neuroprotective factors released by the transplanted cells are responsible for the improvement of RGC function (22, 104).

Unlike stem cell therapies to regenerate the neural retina, therapies to replace the retinal pigment epithelium (RPE), the pigmented cells that function as a retinal blood barrier and metabolically support photoreceptor neurons(105), have been taken to the clinic. These cells have been derived from iPSC and ESC, for which easy and reproducible protocols for RPE differentiation and transplantation have been established (106-108). Initial clinical trials using ESC-derived RPE cells showed that transplanted RPE cells do not abnormally proliferate, form teratomas or trigger graft rejection or any other pathological reactions (109). This subsequently led to the implementation of larger phase I/II clinical trials and have provided evidence of medium to long-term safety and graft survival (34) . However, very little improvement in visual function has been achieved, for which it has been acknowledged that there is the need to implement more rigorous treatment strategies and more accurate clinical testing prior to implementation of these therapies in a large patient cohort (110). RPE cells with neural and mesenchymal progenicity have also been identified in the adult human retina (50), and because of their reported ability to differentiate *in vitro* to form stable RPE monolayers, it has been suggested that they could be used in cell replacement therapies to treat macular disease (111). This approach, however, may not be feasible for autologous or allogeneic human transplantation, because of health and regulatory risks associated with the use of human tissue and the high costs of manufacturing. In addition, the abundant sources of RPE cells easily obtained from pluripotent stem cells surpass the need for the use of cadaveric or autologous RPE cells.

As with any organ transplantation, both, ESC and iPSC express major histocompatibility antigens (112), capable of eliciting immunological reactivity in the recipient. Despite the eye being an immune-privileged site, issues with histocompatibility of the transplanted cells and the recipient need a special consideration. Avoidance of immunoreactivity can be achieved by the use of autologous cells, for which the use of iPSC may provide a practical approach to transplantation. However, due to the high costs of manufacturing a cell line for a single application, the concept of 'cell banks' to maximise the availability of histocompatible cells has gained support over the last few years, and the establishment of cell banks are currently being developed. These will potentially provide cells that can be matched to the major HLA loci including A, B and DR of the transplant recipient to avoid immune rejection.

5. Endogenous regeneration- Targeting factors with potential to induce regeneration

To date, research in the retina regenerative field has focused on cell transplantation to replace damaged neurons in experimental models of retinal degeneration. Despite many adult and pluripotent stem cells being used, a consensus on the most appropriate cell sources for retinal therapies has not yet been achieved. If this research is to be translated into humans there is still uncertainty on the long term cell survival of grafted cells, and concerns with efficacy and allogeneic reactivity are still in need of further investigations. In addition, true regeneration by transplantation has not been yet demonstrated. Based on evidence that quiescent Müller glia with stem cell characteristics are harboured in the adult human retina, a more practical approach to regeneration

in preference to transplantation could involve the stimulation of neural cell renewal by resident Müller cells.

It has been known for a long time that fish and amphibians regenerate the neural retina throughout life (113), however, it was originally thought that warm-blooded vertebrates and mammals were incapable of retinal regeneration. Investigations implicating Müller glia as a source of retinal neurons were first reported in the new born chick (58). Further studies in the rat retina showed that Müller glia could be stimulated to proliferate and differentiate into bipolar cells and rod photoreceptors in response to neurotoxic damage caused by the N-methyl-D-aspartate (NMDA)(60), and that Müller glia in the adult mouse retina could be induced to proliferate and express markers of neural progenitors upon neurotoxic damage and stimulation with growth factors *in vivo* (59). Subsequent investigations of retinal stem cells and their niches in the Tg(*gfap*: GFP) transgenic zebrafish provided compelling evidence for the role of Müller glia as the retinal cells responsible for the regeneration observed in this species (57, 114). These studies demonstrated that in the adult zebrafish Müller glia form the retinal stem cell niche, and that in response to local injury these cells partially and transiently dedifferentiate. During these events, Müller glia re-enter the cell cycle, undergo interkinetic nuclear migration and divide asymmetrically to generate retinal progenitors that give rise to new retinal neurons (57, 114). More recent studies have further shown that newly generated neurons in the zebrafish retina can synaptically re-connect their dendrites and axons, suggesting that new neurons may achieve functional integration in the retina. However, stereotypic synaptic distributions do not resemble those of the original neural population, indicating that developmental cues may be required to achieve true regeneration (115).

Müller glia with stem cell characteristics have been identified in the adult human retina (7, 8), from where they can be isolated and cultured indefinitely *in vitro*. (7). As indicated above, this population of human Müller glia display stem cell characteristics, including unlimited cell renewal, expression of stem cell and neural precursor markers, as well as the potential to differentiate into various retinal neurons *in vitro* (23, 24). In contrast to lower vertebrates, however, these cells lack regenerative capacity *in vivo,* and it can be speculated that this is possibly due to inhibitory factors present in the developed retina to prevent uncontrolled proliferation and tumour formation. Factors produced as a result of inflammatory processes occurring during retina degeneration may also prevent Müller glia from exerting a regenerative function. It would be therefore important to identify these factors in order to develop strategies for the self-repair and regeneration of the diseased retina.

Most human retinal diseases that lead to blindness are characterized by abnormal Müller glia proliferation, which does not lead to repair but to the formation of glial scarring (reactive gliosis) (116). Irrespective of aetiology, gliosis is accompanied by production of inflammatory cytokines such as tumour necrosis factor-α (TNFα), heparin-binding epidermal growth factor (HB-EGF), vascular endothelium growth factor (VEGF) and transforming growth factor- β (TGF-β) (116), and Müller glia itself has been shown to constitute a major source of cytokines and inflammatory factors present in the gliotic retina (117). Although some of these factors have been shown to stimulate the formation of multipotent Müller -derived progenitors in the uninjured retina zebrafish retina and to promote the regenerative response in this species (118, 119) there is no evidence that they have a regenerative role in the human retina. On the contrary, it is possible that these factors may have autocrine effects that may prevent Müller cell progenicity. This is suggested by observations that TGF-β, a cytokine found to be highly upregulated in the gliotic retina, significantly downregulates

the canonical Wnt signalling pathway in Müller glia *in vitro*, inhibiting their differentiation into photoreceptors (120). On this basis, it is reasonable to hypothesize that interaction of pathways involved in neurogenesis and inflammation may affect the ability of Müller glia to endogenously regenerate the diseased retina. Current views favour investigations into the feasibility of activating the potential neurogenesis of these cells in order to regenerate the mammalian retina (121-123) .

Several genes expressed by retinal progenitors are known to be activated in the zebrafish retina in response to injury. One of these genes, the achaete-scute complex-like 1a (ascl1a) factor has been shown to be a key regulator in the generation of Müller glia-derived progenitors responsible for retina regeneration in this species (124). Whilst evidence has been presented that TNFα is produced by dying retinal neurons and that it promotes Ascl1a expression required for Muller glia proliferation in the zebrafish retina (118), there is no suggestion that this cytokine may the same regenerative potential in the mammalian retina. Ascl1 is not expressed in the mammalian Müller glia after injury , but its overexpression in mouse retinal explants *in vitro* and dissociated Müller glia, causes upregulation of retinal progenitor-specific genes, and induces differentiation of these cells into functional retinal neurons that respond to neurotransmitters (125). In addition, overexpression of Ascl1a in the NMDA (N-methyl-d-aspartate) injured retina of young mice, causes Müller glia to differentiate into amacrine and bipolar cells and photoreceptors *in vivo* (126). Further studies in this field indicated that despite Ascl1 overexpression, the neurogenic ability of the mouse retina is lost by postnatal day 16. In addition, lack of neurogenesis was shown to be accompanied by decreased chromatin accessibility in mature Müller glia, suggesting that epigenetic factors limit retina regeneration by Müller glia (127). This was demonstrated by observations that overexpression of Ascl1 by Müller glia in the presence of a histone deacetylase inhibitor, enabled Müller glia in the adult mice to differentiate into cells expressing neural retinal markers, with ability to synapse with host retinal neurons, and respond to light (127).

In view of the latest findings and evidence for the presence of Müller with potential neurogenic ability in the adult human retina, it may be possible that in the non-distant future, new approaches to induce endogenous regeneration of the human neural retina can be developed. This would constitute a preferable alternative to stem cell transplantation currently being investigated, and will transform the lives of millions of people currently affected by retinal degenerative disease.

6. Stem cells as a source of neuroprotective factors to promote neural survival and regeneration

In view of the data shown above, it is possible that stem cell transplantation to repair or regenerate the neural retina may not be aimed solely at replacing neurons, but that delivery of neuroprotective and reparative molecules by stem cells should be viewed as an important factor when developing such therapies. In addition, microglia control with anti-inflammatory agents may need to be considered as an adjunct to cell therapies in order to prolong cell survival and therefore the reparative potential of grafted stem cells within the host retina.

Human neural progenitor cells have been shown to protect photoreceptors, maintain synaptic integrity and support horizontal cell survival when co-cultured with porcine retinal explants (128). Similarly, co-culture of neural progenitors with rat and mice retina *in vitro* have also shown to delay

photoreceptor degeneration (129), and this effect has been attributed to their release of neurotrophic factors. Bone marrow-derived mesenchymal stem cell (MSC) (130)and oligodendrocyte precursors (101) have also been shown to exert a neuroprotective effect on retinal ganglion cells upon transplantation into the eye of rats with experimental glaucoma. In view of the evidence that transplanted stem cells do not integrate into the retina, but that they induce recovery of retinal function, it has been suggested that this effect is due to the stem cell neuroprotective ability. Hence, strategies for cell transplantation as a source of neurotrophins, or direct delivery of recombinant factors into the vitreous have gained much support.

Various approaches to directly deliver retinal neuroprotective agents have been used by many investigators, and have included direct injection of recombinant proteins into the vitreous(131-133), non-viral or viral vector-based delivery of neurotrophin genes (134-136), or cell encapsulation in semipermeable biomaterials for delivery of neuroprotective factors (137, 138). However, the effects of neurotrophins delivered by these methods have been shown to be only transient, and problems can arise by repeated intraocular injections of these factors, as shown by observations that multiple applications of BDNF do not have an additive effect because continuous application of BDNF causes downregulation of its receptor (139). It is not clear whether retinal specific stem cells would be more efficient at promoting endogenous repair and or regeneration of neurons and axonal networks in the degenerated retina, or whether any type of cell with the ability to produce neurotrophins would suffice as a source of trophic factors for retinal repair, and this is subject of current investigations in the field.

Whilst investigations into the nature of neurotrophic factors released by mesenchymal stem cells (MSCs) have identified BDNF and β-NGF as the factors responsible for the neuroprotective effect of these cells when transplanted at sites of nerve injury (140), approaches to enhance the neurotrophic effects of stem cells have also been used. Neural stem cells have been modified to secrete ciliary neurotrophic factor (CNTF), resulting in a significantly increase in RGC survival and axon regeneration upon their intravitreal transplantation in a mouse model of glaucoma (141). Rat and human bone marrow-derived MSCs induced to secrete high levels of BDNF, GDNF and VEGF, exert a marked neuroprotective effect in rat eyes after optic nerve transection (142), whilst MSCs transfected with retrovirus to express high levels of BDNF, release high levels of this protein when transplanted into the axotomised rat retina (143). Based on this evidence, justification for cell transplantation to repair or regenerate the retina may be mainly based on the assumption that cells do not integrate into the diseased tissue, but that neuroprotective factors produced by long lived stem cells within the tissue can maintain residual retinal function without achieving true regeneration.

7. Conclusions and future trends

Although progress on the understanding of the potential and limitations of stem cells has been achieved in recent years, much research is still needed before stem cell therapies can be implemented in the clinic. Encouraging data has demonstrated improvement of visual function after stem cell transplantation into experimental models of retina degeneration. However, neural cell replacement has not yet been achieved, with visual improvement being ascribed to neuroprotective factors released by transplanted cells. Furthermore, evidence that the adult human retina harbours a population Müller glia with stem cell characteristics opens exciting avenues for research into methodologies to induce endogenous regeneration of the retina, as an alternative option to stem cell transplantation.

Figure legends

Fig. 1

The neural retina: Schematic representation of the anatomical localization of the retina and its neural and non-neural cellular components.

Figure 2

Retinal organoid differentiation from ESC and iPSC. Schematic diagram illustrating the formation of retinal organoids from ESC and iPSC cells. (A) Undifferentiated ESC or iPSC, (B) Embryoid body formation at days 2-12, (C) Early protrusion of retinal organoid from embryoid body between days 12-18, (D) Retinal organoid dissociated from undifferentiated cells between days 20-35 (E) Matured organoid between days 40-90 (F) Fluorescence microscopy image of retinal organoid section, stained for the Müller glia cell marker CRALBP.

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Figure 1

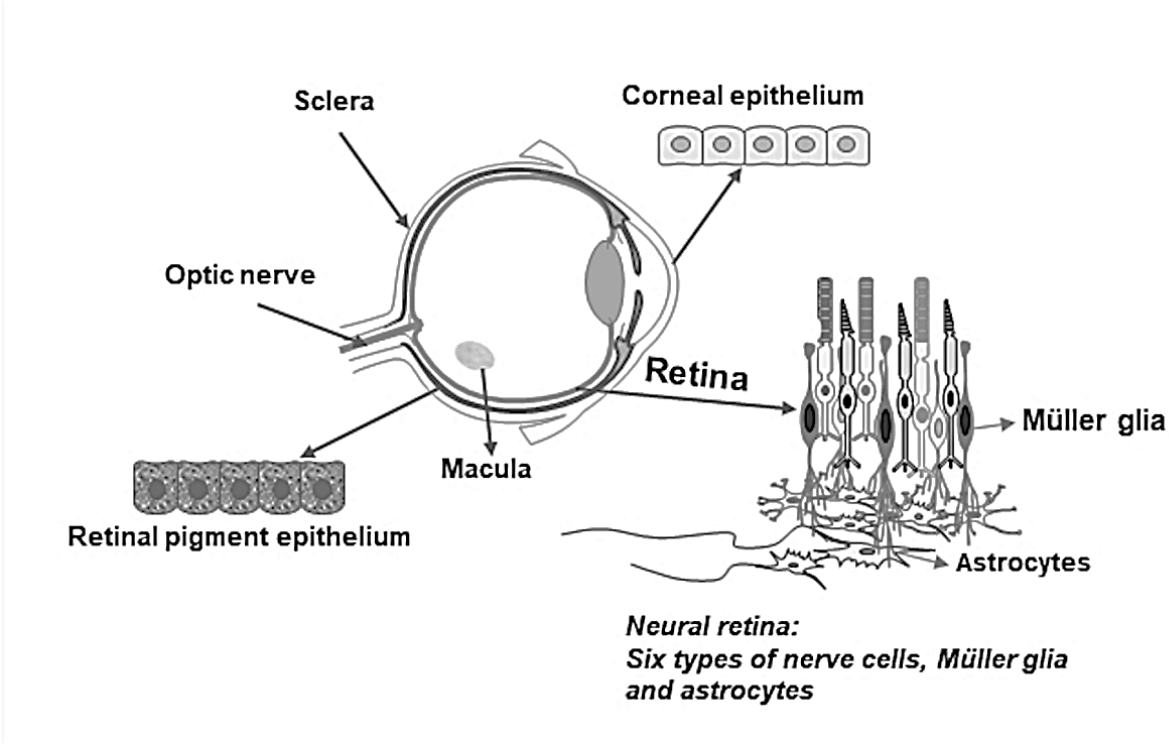
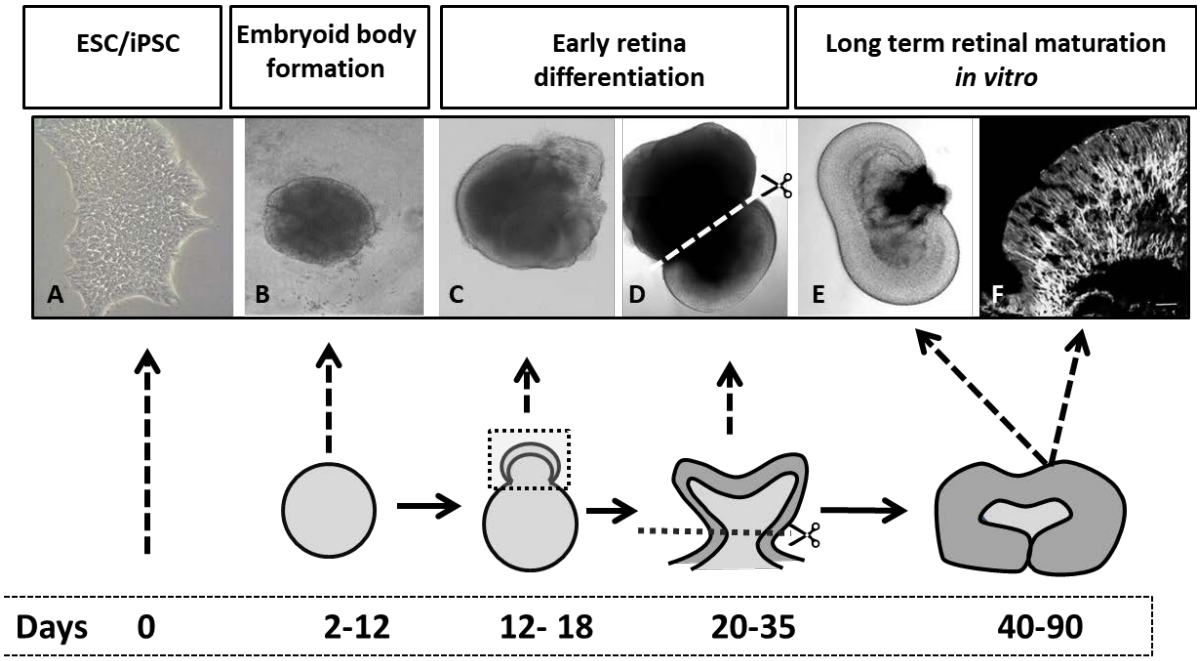

Sclera
Corneal epithelium
Optic nerve
Retina
Müller glia
Macula
Retinal pigment epithelium
Astrocytes
Neural retina:
Six types of nerve cells, Müller glia
and astrocytes

Figure 2


ESC/iPSC
Embryoid body formation
Early retina differentiation
Long term retinal maturation *in vitro*
A
B
C
D
E
F
Days 0
2-12
12- 18
20-35
40-90