Strategies on the application of stem cells based therapies for the treatment of optic neuropathies

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The retinal ganglion cells (RGCs) are not able to regenerate following optic nerve injury resulting in an irreversible vision loss in patients with optic neuropathies including glaucoma. Recent findings in ocular regeneration have opened promising avenues to apply stem cell-based modalities to restore vision in progressive optic neuropathies. Stem cell-based therapies can help to improve retinal regeneration by solving two major problems: (1) by preventing secondary degeneration of RGCs and preserving the remaining vision, and (2) by replacing degenerated RGCs and promoting RGC axon regeneration in the damaged area. The first approach, known as neuroprotective therapy, uses stem cells incorporated into the degenerating retina with an aim to offer a nourishing environment for damaged RGCs resulting in anatomic and functional improvement. The second approach, known as RGC replacement therapy, ultimately aims at replacing the damaged RGCs with healthy RGCs or RGC precursors (Gao et al., 2012; Fu et al., 2019) in order to restore the visual function. Both approaches are graphically represented in **Figure 1**. The implementation of cell replacement therapeutic approaches requires successful generation of clinically safe and functional RGCs in an environment where the transplants survive, appropriately integrate and engraft, as well as establish neurites within the hosts' retina and direct consequent axons towards the relevant regions in the brain (Behtaj et al., 2020). In this work, we discuss the challenges that are required to be addressed prior to the implementation of stem cell-based therapies in clinical practice and, suggest potential solutions to overcome the current limitations.

Stem cell-based RGC neuroprotection strategies: Neuroprotection aims to prevent degeneration of neurons and slow down the progression of vision loss in neurodegenerative diseases such as glaucoma. The application of neurotrophic factors (NTFs) such as brain-derived-, ciliary-, glial cell-derived and nerve growth factor has been a focus of recent investigations as a prominent mean of neuroprotective strategy for it is known that NTFs prevent uncontrolled RGCs loss and aid to the cells survivability. However, the effectiveness of the neuroprotective approach is limited by a relatively short half-life, insufficient permeability and poor concentrations of NTFs in target RGCs.

The use of stem cells as an intraocular slow-release delivery vehicles with the ability of sustainable and multiple NTF-secreting is a known solution to enhance neurotrophic capacity, and consequently, improving the overall efficacy of neuroprotective strategies (Hanumunthadu et al., 2014; Aharony et al., 2017; Boia et al., 2020). As a result, mesenchymal stem cells (MSCs) have been broadly investigated in animal models of neurodegeneration owing to their biological properties, cell expansion potential, immunomodulatory and anti-inflammatory properties, as well as their low propensity towards tumour formation. Additionally, the use of MSCs sidesteps the ethical and legal issues associated with the sourcing, use and application of stem cells from embryos and foetuses since the MSCs are harvested from adult tissues or perinatal derivatives. Both anti-inflammatory cytokines and NTFs secreted by MSCs have been shown to afford neuroprotective capability to enhance RGC survival (Osborne et al., 2018; Boia et al., 2020). However, much uncertainty still exists about the relationship between the populations, sources and types of MSCs and their optimal neuroprotective impacts. Despite the positive results obtained from the application of MSC therapy in animal models by intravitreal MSC injection, this treatment when applied to human subjects yielded limited success (Borkowska-Kuczkowska et al., 2019). One of the most significant drawbacks of using MSCs is that these cells are not able to penetrate to the ganglion cell layer thoroughly and, generally remain attached to the vitreous cavity and the inner limiting membrane (Guymer et al., 2019). The recently introduced cell-free stem cell therapy, which is focused on the application of microvesicles and exosomes released by MSCs, especially bone marrow-derived mesenchymal stem cells, as a novel optical neuropathic treatment modality, may be able to reduce the risks associated with the use of conventional stem cell therapies, specifically, the risk of retinal detachment (Borkowska-Kuczkowska et al., 2019). Applications in which recipient neurons are able to receive genomic material, including messenger ribonucleic acids (mRNAs) and micro ribonucleic acids (miRNAs), transported by these small extracellular vesicles may lead to the activation of target signals and facilitate the re-establishment of intercellular communications. It is important to point out that, protecting RGCs in clinical applications using exosomes derived from bone marrow-derived mesenchymal stem cells requires an efficient delivery of miRNAs, which are responsible for the exosome-mediated neuroprotection (Mead and Tomarev, 2018).

At present, the cell-free stem cell therapy is still at an early stage of development and requires further extensive pre-clinical and clinical studies. Studies that focus on the secretome of bone marrow-derived mesenchymal stem cells, monitor and compare the changes in mRNAs and miRNAs in healthy and damaged RGCs offer a potential to explore neuroprotective signalling pathways and can lead towards the therapeutic application of cell-free stem cell therapies in the nearest future (Mead and Tomarev, 2018).

Stem cell-based RGC replacement strategies: The RGC replacement therapies have been developed as the final resort solution for the treatment of advanced stage of optic neuropathies, since neuroprotective therapies, as they are known at present, are only suitable for limited applications that address the early stages of the disease development in humans (Behtaj et al., 2020). The RGC transplantation, as a treatment modality for retinal regeneration, requires an abundant and a cost-effective source supply of healthy RGCs. Stem cells, including the embryonic stem cells, Müller glial cells as well as the pluripotent stem cells form a viable supply of RGCs. These have been a subject of intense national and international controls as unregulated trade in them could potentially result in precarious biotechnological outcomes. Several research groups have been investigating approaches that would allow an efficient and high yield differentiation of stem cells into functional RGCs by simulating and mimicking the natural environment that the developing eye encounters in an embryonic state, such as by using three-dimensional organoid cultures and a combination of planar and three-dimensional cell growth techniques. Although significant research efforts have been expended towards generating *bonā fidē* retinal neurons from stem cells, majority of these differentiation protocols are not conclusive and yet to be defined towards a high yield production of pure RGCs (Miltner and La Torre, 2018; Fu et al., 2019; Behtaj et al., 2020).

Until now, a number of cell isolation procedures including immunopanning have been introduced as useful approaches for isolation and purification of RGCs from a heterogeneous population of retinal neurons. However, the majority of these purification methods are costly, time consuming and are unable to identify and quantify the low yield of an *in vitro* obtained RGCs (Miltner and La Torre, 2018).

Another limitation associated with RGCs detection is a lack of a unique fingerprint that these cells are able to display. Brn3, Isl1, Thy1, MAP2, RBPMS, and Tuj1 transcription factors, as well as other surface antigens such as CD184 and CD171, are expressed by RGCs and broadly used in research as potential markers for RGC or RGC precursors. However, RGCs with more than forty different sub-types do not display a unique morphological appearance or any specific electrophysiological properties. Therefore, firstly, quality-control standards that can be applied towards definitive classification of RGC sub-types need to be developed, and secondly, based on the known RGC sub-type characteristics, definitive markers, which can be applied to distinguish *in-vitro* generated RGCs from other neurons, have to be identified and developed as well. Another area of consideration is that the ratio of different RGC sub-types for specific RGC replacement therapies needs to be established in order to facilitate successful RGC transplantation (Miltner and La Torre, 2018; Behtaj et al., 2020).

A promising concept in generating *bonā fidē* RGCs is currently offered by CRISPR (clustered regularly interspaced short palindromic repeats), Cas9 (or 'CRISPR-associated protein 9') and TALEN® (transcription activator-like effector nucleases) specific genome editing tools, which are faster, less expensive and more accurate than conventional techniques of editing deoxyribonucleic acid (DNA) and

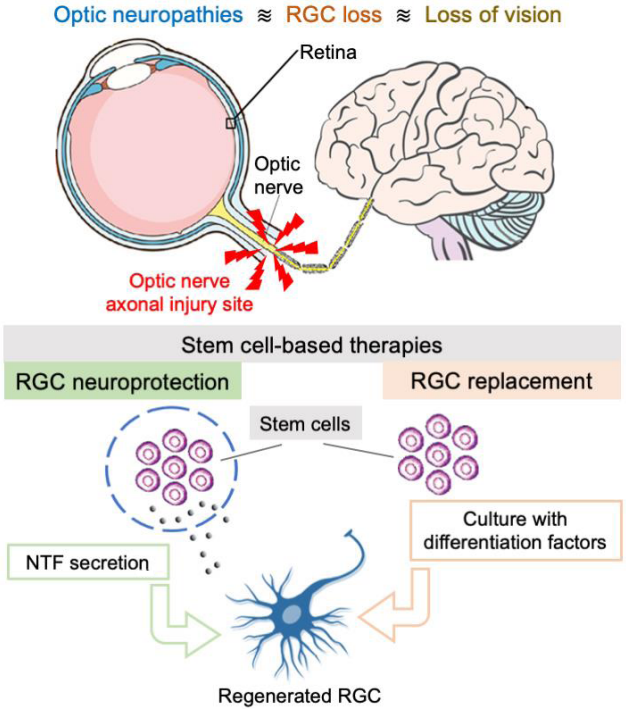

Figure 1 | **The schematics of optic nerve damage in optic neuropathies and stem cell-based therapy approaches facilitating RGC regeneration.**
Optic neuropathy is damage to the optic nerve from any cause, including the loss of RGCs, and is commonly associated with a loss of vision. The damaged RGCs can be regenerated using RGC neuroprotection and RGC replacement treatment modalities, both of which employ stem cells. NTF: Neurotrophic factor; RGCs: retinal ganglion cells.

have a wide range of potential applications. Several pioneering works have confirmed the effectiveness of CRISPR-Cas9 and TALEN® in the detection, sorting and monitoring of functional RGCs, altering DNA sequences and modifying gene function (Nafissi and Foldvari, 2015; Miltner and La Torre, 2018).

Conclusions: Recent research findings focused on the optic nerve neuroprotection have shown that solutions offered by stem cell-based neuroprotective therapies can be highly promising in preventing RGCs degeneration and in preserving the remaining vision. As a result, the use of MSCs owing to their unique properties has been shown to enhance neuroprotective capability and increase RGCs survival. However, a limited capacity of MSCs to effectively penetrate into the ganglion cell layer remains one of the main challenges in the successful application of these stem cells in RGC neuroprotection. The cell-free stem cell therapy has also shown a potential to become a highly efficient stem cell-based neuroprotective protocol in the near future. Before long, stem cell-based RGC transplantation therapies and other modalities could have a widespread clinical application after subsequent neuroprotection treatments, when it has been decisively determined that full RGCs replacement is justified and approved. In this work, an overview of the challenging road ahead to bring the bench results of stem cell-based therapies to the clinic has been provided, highlighting an absence of efficient differentiation protocols for producing clinically safe RGCs in large quantities, as well as the lack of a unique fingerprint that could help to distinguish RGCs from other types of neurons. The evidence shows that genome editing offered by CRISPR-Cas9 and TALEN® tools holds the potential in generating *bonā fidē* RGCs.

Although, while much of the work remains to overcome the aforementioned challenges, the limitations which we alluded to should not detract from recognising the importance of attained intermediate research outcomes, which pave the way to the future conclusive and comprehensive research solutions.

The dream of restoring the visual function in patients with progressive optic neuropathies can be fulfilled by utilising a collaborative effort in which applied pharmacological, bioengineering and/or gene therapy treatment methods have been carefully combined. This combination can offer a consolidated and precise solution of carefully targeted therapies aided by the therapeutic functions of stem cells.

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