Manuscript title: Induced pluripotent stem cells for inherited optic neuropathies – disease modelling and therapeutic development

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Introduction

The inherited optic neuropathies (IONs) cause severe visual impairment with an estimated prevalence of 1:10,000 (1). They encompass a range of genetically diverse disorders characterised by the preferential loss of retinal ganglion cells (RGCs) leading to optic nerve degeneration and irreversible visual loss. Autosomal dominant optic atrophy (DOA) and Leber hereditary optic neuropathy (LHON) are the two most common IONs, sharing overlapping clinical and pathological characteristics despite being genetically distinct conditions (1). About 70% of DOA patients carry variants in the nuclear encoded *OPA1* gene (3q29; OMIM 605290), which encodes for a pro-fusion inner mitochondrial membrane protein. LHON is a primary mitochondrial DNA (mtDNA) disorder and about 90% of patients harbour point variants in *MTND1* (m.3460G>A; OMIM 516000), *MTND4* (m.11778G>A; OMIM 516003), and *MTND6* (m.14484T>C; OMIM 516006), all of which encode for key subunits of the mitochondrial respiratory chain complex I (2-4). Despite the rapidly expanding list of genes that have been identified causing IONs, the pathological hallmark is remarkably similar with early and more severe loss of RGCs within the papillomacular bundle, resulting in a dense central or caecocentral scotoma that accounts for the disabling nature of the visual loss experienced by affected patients (Fig. 1) (3,5) The precise pathways linking genetic variants affecting ubiquitously expressed proteins with preferential RGC loss remains unclear and this lack of mechanistic insight partly accounts for the currently limited treatment options. A key challenge in ION research is the difficulty in obtaining human retinal and optic nerve tissue samples to effectively study the disease. Our understanding of ION disease mechanisms has been derived primarily from non-physiological models, including patient-derived fibroblasts. Although *in vivo* models of DOA and LHON have provided key insights, there are limitations as the animals do not fully manifest the human phenotype in terms of its disease progression (6,7). In the face of these challenges, the advent of induced pluripotent stem cells (iPSCs) is an exciting development for disease modelling,

possessing great translational potential. This review will explore the current advances and future opportunities that iPSC offer in the field of ION research.

iPSC development

Takahasi and Yamanaka (8) revolutionised the disease modelling field through the advent of mouse iPSCs, converting terminally differentiated somatic cells into naïve embryonic stem cell (ESC)-like cells through forced expression of four genes, including *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. Numerous studies followed demonstrating the feasibility of generating human iPSCs (9-12), providing a platform to investigate human disorders that have proven difficult to investigate due to limited access to diseased human tissues, or the unavailability of reliable animal models.

Since their discovery, iPSC research has improved exponentially, enabling the efficient reprogramming of somatic cells using a variety of techniques, including episomal vectors and mRNA constructs, which preserve cellular genomic integrity and increase reprogramming efficiency (13,14). In addition, iPSCs can now be generated using cells acquired from non-invasive methods, such as renal epithelial cells from a urine sample, that are particularly useful for children and in circumstances where it is impractical to acquire a biopsy (15,16). In addition, the generation of iPSCs from adult somatic cells removes many of the religious and ethical concerns associated with ESCs. Importantly, iPSCs, like ESCs, provide a near unlimited source of patient-derived material due to their inherent ability to self-renew (9), whilst also maintaining the pluripotent capacity to generate cells from all three developmental germ layers (17).

Although the potential of iPSCs has made them the ideal tool for disease modelling, several characteristics currently limit their clinical application. Genomic instability that can occur during reprogramming or subsequent culture needs to be avoided, with evidence for large-scale genomic rearrangements, predominantly in chromosomes 8 and 12 (18,19), copy number variations (20,21), and point variants (22-24) occurring in iPSCs. Studies have also demonstrated that iPSCs can retain gene expression and DNA methylation profiles of their somatic cell of origin, in a state known as partially reprogrammed iPSCs, which reduces their differentiation capacity or limits them to cell fates of the germ line of origin (25-28). As such, careful quality control is required to

ensure that the generated iPSCs do not acquire genetic abnormalities before disease modelling or cell replacement therapy.

Current progress in ION modelling

iPSC technology provides a gateway to developing improved, physiologically relevant disease models of IONs (Fig. 2). The modelling of inherited retinal diseases (IRDs) has led the way over the past decade with the derivation of three dimensional (3D) retinal organoids that recapitulate retinogenesis in a spatio-temporal pattern (29-31). Although retinal organoids are at the forefront of IRD research, their use for IONs is limited as they are a heterogeneous retinal cell culture, containing many cells that are unaffected in ION disease progression and only a limited number of RGCs, which are the target cells of interest. Although studies have begun to investigate the effects of disease causing variants on RGC biology using 3D retinal organoids (32), there are consistent reports indicating the loss of RGCs within the inner layers during retinal organoid maturation, likely due to the lack of a terminal synaptic connections or nutrient deprivation. Although this replicates RGC embryonic development (33), it might limit the use of mature retinal organoids as a model of RGC disease (29,31). To counter these problems, several studies have established two dimensional (2D) protocols to generate RGCs (34-39), increasing the specificity and applicability of RGC models, and providing an opportunity to carefully dissect the disease mechanisms driving RGC loss in IONs.

iPSC modelling of LHON

The generation of patient-derived iPSCs, in conjunction with 2D RGC models, has proven useful in studying the pathophysiology of LHON-associated RGC loss. 2D differentiation of iPSCs carrying a homoplasmic double mtDNA variant in *MTND1* (m.4160T>C) and *MTND6* (m.14484T>C) demonstrated that RGCs harbouring these mtDNA variants have significantly increased levels of apoptosis when compared to control and isogenic cybrid RGCs (40). RGCs generated from iPSCs derived from an affected LHON patient harbouring the *MTND4* m.11778G>A variant, alongside an unaffected carrier with the same *MTND4* variant, demonstrated reduced basal respiration and spare respiratory capacity when compared to wild type (WT) control RGCs. Interestingly, the RGCs established from the affected LHON patient exhibited enhanced mitochondrial biogenesis, suggesting a potential compensatory mechanism

to palliate for the reduced bioenergetic output (41). Additionally, RGCs derived from iPSCs carrying the m.11778G>A mtDNA variant demonstrated a range of cellular defects, including increased apoptosis, increased retrograde mitochondrial transport, decreased levels of stationary mitochondria and reduced expression of *KIF5A*, a kinesin required for intracellular organelle transport (42). These studies have brought into focus some of the possible disease mechanisms driven by pathogenic LHON mtDNA variants that eventually lead to RGC death, thus identifying potential therapeutic targets for translational research.

iPSC modelling of DOA

OPA1 is the major causative gene in DOA. There have been numerous studies that have explored the consequences of *OPA1* variants in immortalised cell lines or more accessible human cells, such as fibroblasts and myoblasts (43). To date, a single study has established the effect of an *OPA1* variant in 2D RGC cultures. Patient-derived iPSCs were established harbouring an *OPA1* splice site variant (c.2496+1G>T) that is predicted to cause mis-splicing of *OPA1* transcripts (44). The *OPA1* mutant iPSCs had increased apoptosis and reduced differentiation competence when compared to WT iPSCs, with an inability to form neural progenitor cells (NPCs) that was rescued with noggin supplementation (44). Two studies have reported the generation of mutant iPSCs, carrying the c.1861C>T (p.Q621*) or c.1635C>A (p.S545R) *OPA1* variants, which were derived from patients with a DOA ‘plus’ (DOA+) phenotype (45,46). Although no characterisation of OPA1-related function was conducted, both studies confirmed the differentiation potential of the mutant iPSCs through tri-lineage differentiation assays, providing a platform for further exploring disease mechanisms in DOA.

A subgroup of patients carrying *OPA1* variants will develop DOA+ with more severe neurological features, including cerebellar ataxia, peripheral neuropathy and myopathy, in addition to optic atrophy (47). We are gaining a greater understanding of the impact of *OPA*1 variants on the central nervous system (CNS) with the differentiation of mutant iPSCs into specific neuronal populations (48). One study generated iPSCs from two patients with Parkinson disease carrying a 9 base pair insertion in *OPA1* exon 2, which were subsequently differentiated into dopaminergic neurones (49). These neurones showed accelerated cell death with reduced

mitochondrial oxidative phosphorylation (OXPHOS) and increased mitochondrial fragmentation when compared to WT cells. NPCs and dopaminergic neurons carrying c.1462G>A (p.G488R) or c.1484C> (p.A495V) *OPA1* variants showed significant reductions in mitochondrial OXPHOS and ATP output, and reduced numbers of mitochondria within axonal projections, which also exhibited reduced motility (50,51). Caglayan and colleagues generated an *OPA1* heterozygous knockout ESC line through CRISPR/Cas9 gene editing of WT human ESCs (hESCs) (52). In that particular model, OPA1 haploinsufficiency did not result in significant mitochondrial deficits, but it did inhibit NPC neuronal specification by altering DNA methylation patterns. Further study of *OPA1* variants in iPSC-derived neuronal cell types, other than RGCs, is needed to clarify the mechanisms that contribute to the development of the extraocular features seen in patients with DOA+ phenotypes.

iPSC modelling of syndromic IONs

iPSCs offer an elegant method to create *in vitro* models of the diverse cell types affected in other inherited diseases where optic neuropathy is a prominent feature, such as Wolfram Syndrome (53), Charcot Marie Tooth disease (54) and Friedreich's ataxia (55-57). iPSCs have been generated carrying variants in *WFS1* that account for the majority of cases of Wolfram syndrome. Although there have been no reports of RGCs generated from *WFS1* mutant iPSCs, Shang and colleagues have generated iPSC-derived β-islet cells, which accurately model the pancreatic failure seen in Wolfram syndrome (58).

iPSC therapeutic approaches

iPSC cell therapy

Cell-based regenerative medicine aims to use iPSCs as an autologous or cell banked source to produce specific cell types, which can subsequently be transplanted to replace damaged tissues (59,60). Such an approach is advantageous as it avoids the technical challenges of harvesting neural stem cells (61) and the ethical concerns of ESCs (62). Furthermore, the generation of cells to be transplanted from autologous or closely MHC-matched iPSCs offer the theoretical advantage of minimising the risk of immune rejection and inflammation. The generation of RGCs from iPSCs is now a reality, but the application of this technology to optic neuropathies poses a number of technical challenges that are unique to the anatomical organisation of RGCs and the

precise retinotopic connections that need to be preserved from the optic nerve to the lateral geniculate nucleus (63). The integration of RGCs within the inner retina will need to be optimised and the signalling cues required to guide axonal migration and form the appropriate connections will need to be refined before such an approach can be applied in a clinical setting (64).

So far, there have been no animal trials involving the transplantation of RGCs derived from iPSCs. In a proof-of-concept study, human ESC-derived RGCs were injected into the vitreous cavity and analysed one week later, demonstrating integration of ESC-RGCs into the ganglion cell layer (65). One of the challenges of using iPSC-derived RGCs for optic neuropathies is improving the efficiency of RGC generation, whilst excluding non-RGC differentiation. Furthermore, it is unknown if the integration of non-RGC cell types will have a detrimental effect on the functional and clinical outcomes of transplantation. However, animal models have demonstrated that non-homogenous neural cell populations can integrate into the recipient retina and demonstrate electrophysiological activity (66,67). Promoting the correct retinotopic connections of iPSC-derived RGCs post-implantation is the ideal scenario (68), importantly there is evidence of plasticity within the retinal neural network that could facilitate the integration of iPSC-derived cells into the host retina (69). Nevertheless, the re-establishment of the complex circuitry needed for the proper integration of signals from various pathways in order to achieve a reasonable degree of visual perception remains an important barrier that will need to be overcome (70).

Despite the challenges associated with the transplantation of stem cell derived cells, there have been several clinical trials for outer retinal diseases. Phase 1 and 2 trials involving transplantation of hESC-derived retinal pigment epithelium (RPE) cells in patients with Stargardt macular dystrophy and age-related macular degeneration (AMD) demonstrated significant visual acuity improvement in the treated eyes (71,72). Mandai and colleagues used an iPSC-derived RPE sheet graft in one advanced AMD patient and demonstrated stable vision post-transplantation (73). However, there was evidence of chronic cystoid macula oedema one year following the initial surgery. A second patient was due to be included in this trial, but the transplantation did not proceed due to the identification of genomic aberrations within the patient-derived iPSC, highlighting the importance of stringent quality control for iPSC populations. A number of safety concerns must be considered in any attempts at clinical

transplantation of differentiated cells derived from iPSCs, in particular, the risks of teratogenicity, immunogenicity and genomic instability (74-76). Reassuringly, no long-term safety concerns were raised in the largest stem cell trial that has reported to date and involved 226 patients with spinal cord injuries (77).

iPSC optimisation using gene editing

The use of gene editing technology, such as CRISPR/Cas9, to correct the causative genetic variants in iPSC-derived cells is particularly appealing for monogenic diseases (5). The correction of variants in nuclear genes causing optic atrophy (Fig. 3), such as *OPA1*, offers distinct advantages as the replacement cells will be derived from autologous iPSCs, reducing the chance of immunogenicity and graft rejection. Recent studies have demonstrated the feasibility of utilising CRISPR/Cas9 gene editing to correct variants associated with retinitis pigmentosa (78,79) and Usher syndrome (80). Similarly, CRISPR/Cas9 gene editing has been explored as a potential approach to restore photoreceptor function in Leber congenital amaurosis (LCA), by excising a deep intronic variant in the *CEP290* gene that causes aberrant gene splicing (81). The safety and efficacy of this strategy is being evaluated as part of an ongoing clinical trial (Clinicaltrials.gov NCT03872479).

Although the CRISPR/Cas9 system has revolutionised gene editing, its applicability to editing mtDNA variants, like those associated with LHON, is more challenging (82). Wong and colleagues replaced defective mitochondria from LHON patient-derived cells carrying homoplasmic double mtDNA variants (m.4160T>C and m.14484T>C) with mitochondria from a WT cell line, and this resulted in a decreased susceptibility to apoptosis (Fig. 3) (40). More recently, CRISPR-free mtDNA editing has been established utilising a bacterial cytidine deaminase toxin for targeted manipulation and correction of mtDNA variants (Fig. 3) (83).

iPSC neuroprotective strategies

Until the integration of iPSC-derived RGCs becomes a realistic possibility, stem cell treatment could still offer advantageous therapeutic benefits by promoting neuroprotection with the secretion of trophic factors, such as brain-derived growth factor (BDGF) or platelet-derived growth factor (PDGF) (84). Mesenchymal stem cells (MSCs), which cannot develop into neural tissues, have demonstrated neuroprotective properties in mouse models of optic nerve disease such as glaucoma and traumatic

optic neuropathy (85-87). NPCs derived from iPSCs have also been found to increase RGC survival when transplanted into rats following an optic nerve crush injury (67). This field of research is still in its early stages and we need a much better understanding of the neuroprotective potential of stem cells and how this approach could be optimised for patients with visual loss from IONs.

Drug screening

Successful drug development programmes often require high-throughput screening of thousands of potential therapeutic agents, and this strategy is being applied to neurodegenerative diseases, including Alzheimer disease and amyotrophic lateral sclerosis (88). *In vitro* disease models are advantageous because they can rapidly produce large quantities of target cells, whilst high-throughput readouts, such as 96 or 384 well based assays, increase screening efficiency. For example, one 6 well plate of iPSC-derived RGCs yields the same number of cells (approximately 10 million cells) as the retinal dissection of more than 80 mice (35). This will likely improve with further optimisation and automation of RGC differentiation protocols (89). Differentiated cells from iPSCs have been used in therapeutic screens for ophthalmic diseases, such as nicotinamide in an iPSC model of AMD (54), and antisense oligonucleotide modulation of RNA splicing in LCA (90,91). Although high-throughput screening has not been applied extensively for ION drug development, β-oestrogen was found to decrease apoptosis in iPSCs harbouring *OPA1* variants, demonstrating the applicability of such an approach if the proper readouts are used (44). The trilineage differentiation potential of iPSCs is also a major asset for drug screening as they are able to produce a wide range of cell types, including motor neurones and myocytes, which can be affected in patients with more severe syndromic IONs (92-95).

There is no doubt that 2D RGC models are efficient tools to study both the therapeutic and toxic effects of drug molecules (96,97), however, they lack the complex cell-cell interactions seen in the native retina and optic nerve. Ideally, this initial stage of drug screening could then be further substantiated within 3D retinal organoids, which more closely match the physiological retinal niche, providing further *in vitro* evidence before transitioning into more costly *in vivo* animal model studies (98). Importantly, the use of both 2D and 3D iPSC ION models can facilitate the rapid development of therapeutic strategies, providing a gateway to move into early phase clinical trials.

Conclusion

IONs cause severe permanent visual loss and they represent a major societal burden given their onset in childhood and young adulthood. Although still in its early phase, the application of iPSCs to the field of ION research has tremendous potential both for disease modelling and as a powerful tool for therapeutic drug development and genomic medicine. In the short-term, iPSCs provide efficient models that can be applied to study different causative genes and variants, which is a major advantage given that IONs are genetically heterogeneous. Furthermore, CRISPR/Cas9 correction of disease-causing variants in iPSCs offer the ideal controls to better distinguish genotype-phenotype relations *in vitro*. The optimisation of RGC differentiation protocols will increase the scalability of iPSC-derived RGCs for drug screening, not only to assess efficacy, but to exclude possible toxic effects. In the medium-term, iPSCs offer the ability to introduce autologous cells within the retina, which may convey neuroprotective effects, whilst reducing potential adverse immune responses. In the long-term, iPSCs combined with gene editing technology could be employed for RGC replacement to rescue vision in patients with more advanced optic nerve degeneration. IONs are ideal targets for iPSC-based therapeutics and the future looks bright in terms of much long-awaited breakthroughs for these blinding diseases.

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Figure legends

FIG. 1. Pattern of retinal degeneration in inherited optic neuropathies.

(A) The axons of retinal ganglion cells (RGC) constitute the retinal nerve fibre layer (RNFL) and they converge, exiting the eye as the optic nerve. **(B)** In patients with inherited optic neuropathies (IONs), preferential degeneration of RGCs occurs resulting in thinning of the RNFL, the development of optic atrophy and progressive irreversible visual decline **(B)**.

FIG. 2. Derivation of retinal ganglion cells from patient-derived somatic cells.

Schematic overview of induced pluripotent stem cell (iPSC) and RGC generation from somatic cells, such as skin cells, collected from patients with visual loss from an ION. The somatic cells are reprogrammed towards iPSCs using non-integrative reprogramming and gene editing technologies. The iPSCs are characterised and expanded before being subjected to *in vitro* differentiation of RGCs through either 3D retina organoids or direct RGC generation through 2D protocols. *In vitro* RGCs provide a key resource for investigating genetic variant associated disease mechanisms, therapeutic screening and potential cell replacement therapies.

FIG. 3. Schematic overview of gene editing strategies to correct the underlying genetic variants in patient-derived iPSC populations.

CRISPR/Cas9 gene correction - CRISPR/Cas9 gene editing can be utilised to edit nuclear encoded variants (purple bars), combined with homology directed repair (HDR) that utilises a repair template encoding the desired base changes (orange bars) to correct the disease-causing variants.

Mitochondrial replacement – The mutant mitochondrial DNA (mtDNA) (red mitochondria) in LHON patient-derived iPSCs are depleted and then replaced with mitochondria carrying wild-type mtDNA (green mitochondria) to restore mitochondrial genome integrity and function.

mtDNA gene correction - Defective mitochondria (yellow mitochondria) harbouring mtDNA variants (yellow mitochondria) can be corrected utilising bacterial-derived cytidine deaminase toxins resulting in respiratory competent mitochondria with wild-type mtDNA (blue mitochondria).

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