

Published in final edited form as:

Biochem Soc Trans. 2016 October 15; 44(5): 1245–1251. doi:10.1042/BST20160156.

Using induced pluripotent stem cells to understand retinal ciliopathy disease mechanisms and develop therapies

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Abstract

The photoreceptor cells in the retina have a highly specialized sensory cilium, the outer segment, which is important for detecting light. Mutations in cilia related genes often result in retinal degeneration. The ability to reprogram human cells into induced pluripotent stem cells (iPSCs) and then differentiate them into a wide range of different cell types has revolutionized our ability to study human disease. To date, however, the challenge of producing fully differentiated photoreceptors *in vitro* has limited the application of this technology in studying retinal degeneration. In this review we will discuss recent advances in stem cell technology and photoreceptor differentiation. In particular, the development of photoreceptors with rudimentary outer segments that can be used to understand disease mechanisms and as an important model to test potential new therapies for inherited retinal ciliopathies.

Introduction: the retina and specialized cilia

The retina is responsible for transducing light into an electrochemical signal and transmitting that signal to the brain. It is comprised of around 55 neuronal subtypes, which all have different functions to allow the propagation of the light signal [1]. The cells in the retina that detect light are the photoreceptors, consisting of two types: cones and rods. Cones are responsible for daytime and colour vision in humans, whereas rod cells detect much lower levels of light and are therefore important for night vision. Photoreceptors possess a highly specialised sensory cilium known as the outer segment (OS). The OS consists of tightly packed membranous discs laden with photoresponsive opsin proteins as well as other components of the phototransduction machinery. The OS is joined to the biosynthetic inner segment (IS), which contains the endoplasmic reticulum (ER), by the connecting cilium (CC), a specialized cilia transition zone. The apical 10% of rod OS discs are shed daily and phagocytosed by the overlying retinal pigment epithelium (RPE) [2]. All the components needed for the assembly, maintenance and continuous turnover of the OS are synthesised in the IS and transported through the CC via the intraflagellar transport (IFT) machinery [3].

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Cilia are microtubule-based organelles that project from the surface of eukaryotic cells. They are present from the earliest stages of development through to highly differentiated cells in adult tissues. Once thought to be a vestigial organelle they are now known to have an important function as sensors relaying information from the extracellular environment. Ciliopathies are a relatively recently defined class of inherited disorders that involve aberrant ciliary function [4]. The clinical manifestations of this dysfunction range from single organ involvement, such as retinitis pigmentosa (RP) to multi-organ involvement and syndromic disease, affecting a broad range of organs including the central nervous system, kidneys and adipose tissue [5]. Ciliopathy genes encode structural or functional components of the cilia or their basal bodies. Perhaps as a consequence of the high protein-trafficking load through the photoreceptor CC, the retina is possibly the most commonly affected organ [6].

Photoreceptor degeneration is a major cause of blindness. There are over 250 different causative genes identified to date (RetNet; <https://sph.uth.edu/retnet/>). Forms of inherited retinal dystrophy include RP and Leber congenital amaurosis (LCA). RP is a group of heterogeneous retinal dystrophies that usually involves progressive rod cell degeneration with secondary cone death. RP affects around 1:4000 individuals worldwide, leading to 1.5 million affected patients [7]. RP often does not present until the second decade; however X-linked forms of the disease can result in early onset degeneration. For example, mutations in the cilia-associated gene *RP2* (retinitis pigmentosa 2; OMIM 312600) result in X-linked RP [8]. In addition to the rod degeneration usually seen in RP patients, some *RP2* patients may also have a macular atrophy leading to decreased visual acuity in childhood [9]. LCA is an early onset retinal degeneration characterised by profound visual impairment or loss in early childhood [10]. LCA accounts for 5% of all inherited retinal dystrophies and affects up to 1:30000 patients [10]. LCA is also highly heterogeneous; there are currently 13 known genetic loci for LCA (RetNet) affecting different retinal functions. One of the most commonly mutated genes is *CEP290* (centrosomal protein of 290kDa; OMIM 611755), accounting for around 15-25% of LCA cases [11]. Here, we will mainly focus on *RP2* and *CEP290* retinal ciliopathies.

RP2 and CEP290

RP2 is a GTPase activating protein (GAP) for the essential cilia-related GTPase, Arl3 [12,13]. *RP2* is a ubiquitously expressed protein; in the retina it is predominantly localized at the plasma membrane [14]. However there is a pool of *RP2* that localizes to the basal body of the cilia in photoreceptors, suggesting a role in cilia protein trafficking [14,15]. Mutations in *RP2* account for approximately 15% of X-linked RP cases [8]. In particular, nonsense mutations are relatively frequent in *RP2* patients, such as the R120X mutation [16].

Animal models of *RP2* have reported conflicting phenotypes. For example, morpholino knockdown of *RP2* in zebrafish produces a range of severe developmental eye defects, from small eyes to delayed/defective retinal lamination and photoreceptors with no outer segments and multiple systemic developmental defects [17–19]. However, an *RP2* knockout zebrafish had mild retinal degeneration associated with disruption of GRK1 and rod transducin, but displayed no overt systemic phenotype [20]. *RP2* knockout mouse models

have produced some conflicting retinal phenotypes as well; cone opsin mis-localisation was observed in one model [21], whereas normal cone opsin traffic but abnormal traffic of prenylated phototransduction proteins PDE6- γ and GRK1 was observed in another [22]. Given the range of conflicting phenotypes reported in animal models, investigating actual human RP2 mutations in retinal cells derived from patients provides a new opportunity to gain insight into human disease mechanisms.

CEP290 is a protein found in all ciliated cells at the transition zone between the basal body and axoneme, and is essential for ciliogenesis [23,24]. A recent study utilising super-resolution microscopy located CEP290 as a hub on which other transition zone proteins assemble and postulated that these are involved in the formation of Y-links bridging the ciliary membrane and the axoneme [25]. Mutations in *CEP290* are associated with numerous syndromic ciliopathies, yet non-syndromic LCA is the most common outcome [26,27]. The majority of CEP290 LCA patients (86%) have at least one copy of the most common CEP290 mutation, a deep intronic c.2991+1665A>G change which leads to mis-splicing and inclusion of a stop-codon containing cryptic exon in CEP290 mRNA [27,28]. There is still some correct splicing of exons 26 to 27, such that this intronic mutation leads to reduced CEP290 protein levels and behaves as a hypomorph.

The *rd16* mouse model contains an inframe deletion in *Cep290* that produces a truncated protein. *rd16* mice have considerable deterioration of rod and cone function at p18 a reduced outer nuclear layer and OS degeneration [23]. A *Cep290* knock out (null) mouse has no OS formation and no surviving photoreceptors at p28, the majority of these knockouts died of hydrocephalus [6]. A humanised mouse model of the CEP290 deep intronic mutation resulted in the creation of a separate, distinct cryptic exon not seen in human cells [29], highlighting the difficulties of modelling some mutations in mice to investigate human disease mechanisms. Therefore, differentiating patient induced pluripotent stem cells (iPSCs) to photoreceptors may be the only route by which to recapitulate the mechanisms of CEP290 LCA.

Using iPSCs to model retinal degeneration

Much recent work has focused on the role of iPSCs as a model of disease and as a potential route for therapy. Stem cells are an appealing approach to disease modelling, due to their pluripotent nature that allows them to differentiate into multiple cell types, coupled to the presence of the disease associated changes in the correct genomic and cellular context [30]. iPSCs were first described in 2006 and have since been used as models in a plethora of diseases [31,32]. Indeed, the generation of iPSCs and subsequent differentiation into retinal cell types from patients allows the study of mutations causing retinal degeneration in a human cell and mutation-specific paradigm [33]. Initial methods focused on creating pure, single cell-type cultures; for example, many studies have concentrated on differentiating iPSCs into RPE cells, which can be achieved in a two-dimensional culture system [34–36]. This approach has successfully resulted in insights into disorders affecting the RPE; for example, RPE cells have been differentiated from patients with mutations in *MERTK* [37], *RPE65* [38], *BEST1* [39], and *OATI* [40]. Retinal-progenitor and rod-like cells have been created from RP patients with mutations in the rhodopsin gene that were reported to

recapitulate the disease phenotype; however, these progenitor cells were developmentally too immature to have OS containing rhodopsin [41,42].

3D 'organoids'

Increasingly researchers are using three-dimensional organoid models derived from iPSCs to model disease tissue [43]. Organoid culture from iPSCs allows the creation of multiple cell-types in a more natural, organised cellular environment [44,45]. The retina has been successfully modelled in this way [46,47]. Multiple groups have achieved the generation of self-organising three-dimensional optic cup structures derived from human embryonic stem cells (ESCs) and iPSCs that develop cells with all the characteristics of photoreceptor precursors [48–50]. This technology will allow greater insight into the consequence of retinal-dystrophy mutations in highly specific cellular and genetic context. However, these studies did not produce photoreceptors with morphologically distinct OS, limiting their application to study retinal ciliopathies.

Transplantation into a host retinal context appears to enhance the differentiation of these organoid produced photoreceptor progenitors, as shown with mouse ESC [51] or human iPSC [52]. Patient iPSC-derived optic cup-like structures and their transplantation were used to probe a novel intronic mutation in *USH2A* that causes RP [52]. Recently, we have used these methods of 3D optic cup differentiation to produce photoreceptors with morphologically distinct rudimentary OS *in vitro*, without the need for transplantation [53]. These iPSC-derived OS take up to 20 weeks to develop and express rod and cone opsins ([53]; Figure 1). Furthermore, the CC can be visualised with a variety of cilia markers. Therefore, we can now use these 3D optic cups to probe retinal ciliopathy disease mechanisms and test potential therapies.

Retinal disease mechanisms and therapeutic development using iPSCs

Advances in gene-editing technology, such as the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, could allow for the correction of gene defects before organoid culture and potentially individual cell replacement therapy in the ultimate personalised medicine [54]. iPSC-derived disease cells can also be used to test small molecule based approaches that are either mutation or gene specific that cannot easily be tested in animal models. For example, translational read-through inducing drugs (TRIDs) that read-through premature termination mutations in a range of genes, or antisense oligonucleotides (AONs) to correct mis-splicing events require the specific gene and cellular context that iPSC-derived cells and organoids can provide.

We have used X-linked RP patient-iPSCs to produce RPE cells with the *RP2*R120X mutation, which results in a premature stop and loss of RP2 protein from the basal body [55]. The loss of RP2 had no effect on cilia length or incidence, but IFT20 was dispersed in the RP2 patient RPE, suggesting RP2 may be involved with the cilia-based trafficking of a specific group of cilia proteins. Indeed, the traffic and membrane association of the beta subunit of transducin (Gβ1) was also disrupted [55,56]. Importantly, we were able to reverse

these phenotypes using TRIDs, by restoring the RP2 protein levels up to 20% of normal in the RPE cells [55].

More recently, we used patient-derived iPSCs to model CEP290-LCA. The intronic c.2291+1665A>G mutation leads to a premature stop codon resulting in reduction in protein levels, although crucially there are residual amounts of wild-type mRNA leading to some full length protein expression [27]. The mis-splicing of CEP290 leads to reduced ciliation of cells and those cilia that are still present are shorter, even in fibroblasts, despite the expression of 40-50% of the correct wild-type exon 26-27 transcript [27,53,57]. Nevertheless, why a reduction in CEP290 of approximately 50% (the same that is present in carriers of null mutations that have no retinal phenotype) leads to LCA is unclear. To resolve this paradox we differentiated homozygous c.2291+1665A>G patient iPSC into RPE and 3D optic cups and compared cryptic splicing levels between cell types. The CEP290-LCA iPSC had reduced cilia incidence, but this did not affect their differentiation, which is expected given the phenotype of LCA but perhaps surprising given the importance of cilia for signalling during development. The comparison of the different cell types showed that optic cups had the highest levels of aberrant splicing and cilia defects, indicating that the retina has a much greater reduction of CEP290 levels than other tissues, including RPE, suggesting this is the underlying reason for LCA and not syndromic disease [53]. Interestingly, the increase in mis-spliced CEP290 coincided with photoreceptor differentiation and the splicing of other photoreceptor specific exons. RNA-Seq of human retina suggests that photoreceptors have exceptionally high levels of alternative exon usage [58] and the increased aberrant splicing in CEP290 might be a direct consequence of this attempt to maximise their genome.

CEP290-LCA iPSC retinal progenitors have also been used to test the potential of a gene replacement approach for CEP290 [59]. This study used lentivirus, as it has a higher packaging capacity compared to adeno-associated virus (AAV), which cannot package a large gene like CEP290 (8kb). The transduction of patient retinal progenitor cells with full-length-CEP290 lentivirus restored CEP290 mRNA expression, suggesting this approach might be potentially beneficial for patients in the future [59].

AON technology has previously been shown to reduce aberrant splicing in CEP290-LCA fibroblasts, leading to an increase in cilia incidence and cilia length in patient cells [57,60]. Moreover, a recent study assessed the AAV-mediated delivery of AON to the humanized mouse model of CEP290-LCA [61]. The AAV-CEP290 AON resulted in corrected splicing and decrease in the cryptic exon formation in the mouse retina [61].

We tested an AON approach in CEP290-LCA optic cups using an antisense morpholino to the cryptic splice site in *CEP290* c.2291+1665A>G (CEP290-MO). Treatment with CEP290-MO reduced mis-splicing and increased the level of normal transcripts to around 50%, which led to increased ciliation, cilia length and improvement of the cilia trafficking of essential photoreceptor cilia proteins, such as RPGR [53]. This study highlights further the potential of using 3D organoids to produce human retinal cells in an appropriate cellular environment to model the effects of gene-specific mutations.

Collectively, these data and experimental models highlight that iPSC derived retinal cells offer a new paradigm to study retinal ciliopathy disease mechanisms and test potential therapies. Moreover, given that these proof of concept studies can show efficacy and safety in the correct human target cells they also provide the hope of a rapid translation to the clinic.

Acknowledgements

This research is supported by The Guide Dogs for the Blind, Fight for Sight, Moorfields Eye Charity, the Rosetrees Trust, the London Project to Cure Blindness, the Special Trustees of Moorfields Eye Hospital, The Wellcome Trust and National Institute for Health Research (NIHR) Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology.

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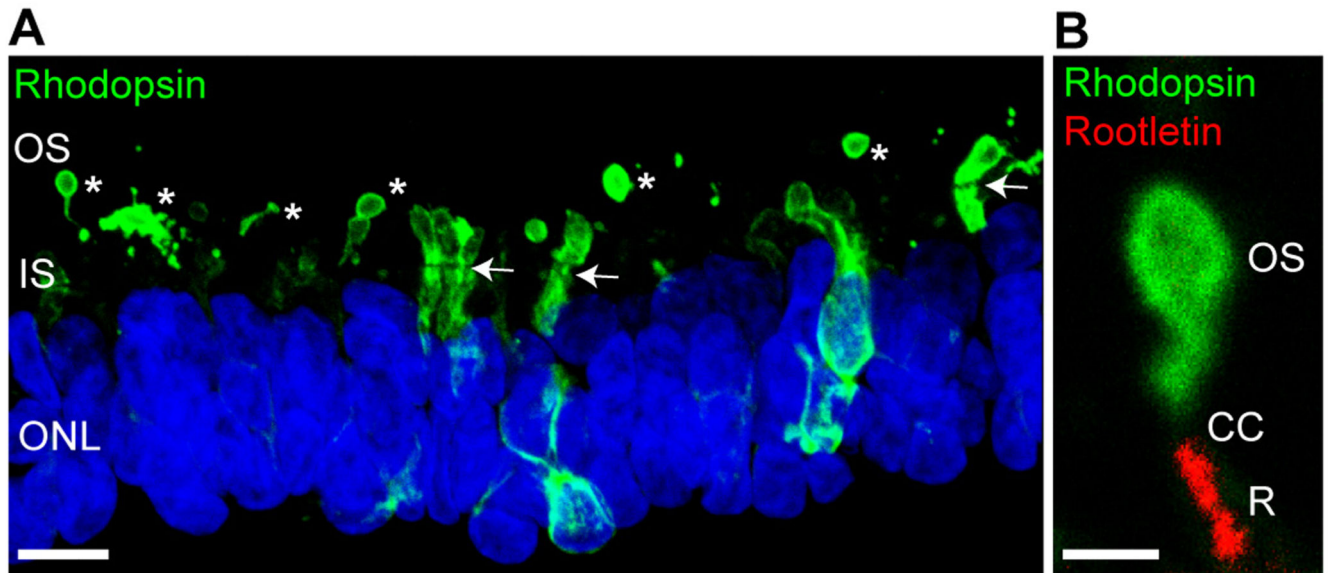


Figure 1. Rudimentary outer segments in iPSC 3D organoid optic cups.

(A) Rhodopsin localization (green) in 145 day old control iPSC derived optic cups. Rhodopsin immunoreactivity is most intense in the rudimentary outer segments (OS, and *). The inner segments (IS and separated from the outer nuclear layer stained with DAPI (blue) by the outer limiting membrane (OLM; highlighted with arrows). Immature photoreceptors have still have strong immunoreactivity in the IS and ONL. Note the rudimentary OS tend to break during processing. (B) Magnified OS stained with rhodopsin (green), the base of the cilium is highlighted with rootletin (red, R) highlighting the connecting cilium (CC) between the two. Scale bars = 10 μ m (A) and 2 μ m (B)