Editorial

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Unraveling the mysteries of pre-mRNA splicing in the retina via stem cell technology

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With mutations in more than 200 different genes identified, inherited retinal diseases (IRDs) represent one of the most heterogeneous disorders in humans, both genetically and clinically (https://sph.uth.edu/retnet/). Typically, IRDs are progressive in nature, and often result in patients becoming legally blind, although the age at which this occurs can vary enormously, from early childhood to late adulthood. Long considered incurable diseases, technological innovation has tremendously boosted the development of therapeutic strategies for IRD over the last decade, especially in the field of gene therapy. Pioneer successes have been achieved by delivering recombinant adeno-associated viruses (AAVs) harboring a wild-type copy of *RPE65* cDNA to the retina of patients with mutations in this gene (1-4). More recently, similar approaches have been used to treat patients with mutations in *CHM* (5) or *MERTK* (6). Alternative strategies that have been tested in clinical trials include oral delivery of a vitamin A derivative 9-cis-retinyl acetate in patients with mutations in *LRAT* or *RPE65* (7). Overall, all clinical studies conducted so far have reported on acceptable safety profiles in all subjects, and moderate efficacy in several patients.

Prior to the initiation of clinical trials in humans, preclinical efficacy needs to be proven in cellular and/or animal models. Although some genes associated with IRD are broadly expressed, many others show expression that is restricted to the retina, complicating *in vitro* studies. As recently reviewed by us, a large variety of mutant animal models for IRD exist, many of which have been successfully employed to demonstrate preclinical efficacy of therapeutic interventions (8). Yet, in some cases, the animal's phenotype does not correspond to that observed in humans with mutations in the same gene. In addition, animal studies are usually expensive and time-consuming, especially in those species that more closely resemble humans in terms of ocular anatomy and evolutionary genetics.

With the prominent discovery of induced pluripotent stem cells (iPSCs) in 2006 (9), the stem cell technology field rapidly emerged thereafter, and many scientists have successfully attempted to use these iPSCs for the differentiation towards their cell type / tissue of interest. In terms of the retina, various groups succeeded in establishing protocols to generate human iPSC-derived photoreceptor-like cells, including those from patients with IRD (10-12). This not only provided means to study the pathophysiological mechanisms underlying IRD in a cell relevant to the phenotype within the proper genetic context, but also allowed to assess the preclinical efficacy of novel therapeutic interventions (13,14).

In the June 2016 issue of *Cell Stem Cell*, David Parfitt and colleagues employed iPSC technology to study the molecular mechanisms underlying a specific genetic subtype of *CEP290*-associated Leber congenital amaurosis (LCA, the most severe subtype of IRD) and to assess the efficacy of antisense oligonucleotides (AONs) to rescue the splice defects that are associated with this disease (15). There are two important highlights to extract from this paper, namely the suitability of iPSC-derived optic cups to study disease mechanisms and test novel treatment strategies, as well as the discovery of a cell-type specific distribution of aberrantly *vs.* correctly spliced *CEP290* transcripts.

Almost a decade ago, the most recurrent mutation underlying LCA was identified, namely a deep-intronic

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variant c.2991+1655A>G in *CEP290* (16). *CEP290* is comprised of 54 exons and encodes a 2,479 amino acid protein localized in the centrosome and basal body of cilia (17). It is thought that CEP290 plays an important role in cilium assembly as well as ciliary protein trafficking (17,18). The c.2991+1655A>G mutation, present in up to 15% of all LCA cases in several North-American and European countries (16,19-21), creates a cryptic splice donor site in intron 26 and results in the inclusion of a 128-bp pseudoexon harboring a premature stop codon to some but not all *CEP290* transcripts. We and others have shown that, in lymphoblastoid cell lines or in fibroblasts from LCA patients homozygously carrying the deep-intronic *CEP290* mutation, administration of AONs redirected normal *CEP290* splicing, significantly increased CEP290 protein levels, and fully rescued a ciliary phenotype (less ciliated cells and shorter cilia) present in these cells (22-24). The broad expression profile of *CEP290* in many cells of the human body allowed the use of these easily accessible cells for initial intervention studies. Yet, although CEP290 itself may be broadly expressed, several of the proteins it interacts with are uniquely or predominantly expressed in the retina. Therefore, to determine whether restoration of *CEP290* splicing and CEP290 protein synthesis also results in re-establishing protein trafficking at the connecting cilium within the photoreceptor cell, a model system more relevant to the disease was needed. Our own attempts to mimic the pathophysiology associated with the c.2991+1655A>G mutation in a transgenic humanized mutant *Cep290* mouse model failed, due to species-dependent differences in the recognition of splice site sequences (25,26). By employing patient-derived three-dimensional optic cup organoids, Parfitt *et al.* have now shown that restoration of aberrant *CEP290* splicing in photoreceptor cells not only restores the structure of the cilium but also rescues ciliary trafficking of RPGR and Rab8, two important ciliary proteins within photoreceptor cells. It should be noted that the degree of splice correction in the iPSC-derived optic cups appeared to be somewhat less compared to previously published studies employing lymphoblastoid and fibroblast cells (22-24). This can be either due to the fact that Parfitt and colleagues employed morpholinos compared to 2'-O-methyl AONs with a phosphorothioate backbone used in the other studies, or that the three-dimensional optic cups are somewhat more difficult to transfect compared to adherent cells or cells in suspension. Nevertheless, the data generated by Parfitt *et al.* illustrate the unique opportunity that iPSC-derived optic cups provide to study the molecular characteristics of IRD as well as the preclinical efficacy of novel therapeutic interventions.

A second, very intriguing, discovery by Parfitt *et al.* is the observation that the relative amount of aberrantly spliced *CEP290* due to the c.2991+1655A>G mutation appears to be significantly higher in iPSC-derived optic cups compared to other cell types, thereby answering a long-standing question on the molecular mechanism underlying *CEP290*-associated LCA. Ever since the discovery of the deep-intronic *CEP290* mutation, it has been largely associated with recessive non-syndromic LCA (16,20,21,27), whereas a plethora of other, full loss-of-function *CEP290* alleles result in more severe, syndromic phenotypes such as Meckel or Meckel-Gruber syndrome, Joubert syndrome and Senior-Løken syndrome (28-30), all in an autosomal recessive manner. Given the initial observation that in lymphoblast and fibroblast cells from patients homozygous for the c.2991+1655A>G mutation, approximately 50% of all *CEP290* transcripts were normal (without the 128-bp pseudoexon inclusion), the question arose why these patients were suffering from congenital blindness, whereas parents from patients with other *CEP290*-associated ciliopathies did not show any signs of visual impairment at all, despite the fact that they are also predicted to have only ~50% of wild-type *CEP290* expression. Different hypotheses existed to explain this phenomenon, including a retina-specific 'dominant-negative' or 'gain-of-function' effect exerted by the N-terminal protein product resulting from premature termination of CEP290 protein synthesis, or a different ratio of aberrantly *vs.* correctly spliced *CEP290* in retinal compared to other tissues. The latter hypothesis has now been supported by the observation that in the iPSC-derived optic cups from LCA patients with the deep-intronic *CEP290* mutation, the amount of aberrantly spliced *CEP290* harboring the pseudoexon was significantly more abundant than the wild-type mRNA, in contrast to the ~1:1 ratio observed in lymphoblasts and fibroblasts. Of particular interest, in iPSC-derived retinal pigment epithelium from the same LCA patients, the ratio was more similar to the one in fibroblasts, indicating that, even within the retina, the differential inclusion of the pseudoexon is a cell type-specific event.

Ever since the identification of mutations in genes encoding ubiquitously expressed splicing factors such as *PRPF3*, *PRPF6*, *PRPF8* and *PRPF31* (31-34) to underlie autosomal dominant retinitis pigmentosa (RP), an intriguing relationship between pre-mRNA splicing and photoreceptor function exists. Possible explanations for

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the fact that mutations in these genes only give rise to a retinal phenotype include a particularly high metabolic demand of photoreceptor cells, specific aberrant splicing of genes crucial for retinal function, or additional, retina-specific functions of these proteins besides pre-mRNA splicing. In many efforts to deepen our understanding of the relationship between splicing and photoreceptor function, more recent studies have revealed additional insights, for instance by the observation that the human retina displays an enormous degree of splicing diversity (35), the identification of retina-specific isoforms of certain genes mutated in syndromic and non-syndromic IRD (36-38), or the discovery of genetic modifier alleles that influence the penetrance of certain genetic subtypes of RP (39). The findings by Parfitt *et al.* further illustrate the fascinating aspects of pre-mRNA splicing in photoreceptor cells, and its relationship to photoreceptor dysfunction and disease.

What it comes down to is that apparently, the splicing machinery that exists in photoreceptor cells is essentially different compared to that in other cells of our body. That raises two important questions: (I) what are the exact splice factors crucial for the photoreceptor-specific recognition of splice sites and exons; and (II) what are the consequences of this for the molecular diagnostics of IRD? A very recent study by Murphy *et al.* has begun to answer the first question, and revealed intriguing new insights into photoreceptor-specific splicing, albeit in mouse. The authors found that several genes, including *CEP290*, harbor exons that display a high degree of inclusion in photoreceptor cell transcripts while being almost completely absent in other cells. In addition, it appears that a protein called Musashi 1 plays a key role in the recognition and subsequent splice regulation of these particular exons in photoreceptor cells (40). So how does this affect the molecular diagnostics of IRD? Following the discovery of the deep-intronic c.2911+1655A>G mutation in *CEP290* (16), several other deep-intronic mutations leading to pseudoexon insertion and underlying non-syndromic IRD have been discovered, for instance in *ABCA4* and *OFD1* (41-44). In a number of these examples, the insertion of the pseudoexon was not observed in all transcripts of the corresponding gene, at least not in the peripheral blood cells or keratinocytes of the patients that were used in these studies. This leads to believe that in these cases, the inclusion of the corresponding pseudoexon may also be much more prominent in photoreceptor cells compared to other cells of our human body, similar to the deep-intronic mutation in *CEP290*. This also means that genetic variants identified in exome or whole genome sequencing studies, either exonic or intronic, may somehow affect pre-mRNA splicing of the corresponding gene specifically in photoreceptor cells, and thereby be misinterpreted by bioinformatic prediction programs or even by *in vitro* assays using non-retinal cells. Only by a combination of employing model systems such as the three-dimensional optic cup organoids, the discovery of key splice factors like Musashi 1 that regulate photoreceptor-specific splicing, and collecting large whole genome and retina-specific transcriptome sequencing datasets, we can increase our understanding on the relationship between genetic variation and photoreceptor-specific pre-mRNA splicing in the near future.

Taken together, the iPSC-derived optic cup model system as developed by the Cheetham lab, amongst others, provides us with unique opportunities to study the molecular mechanisms underlying IRD, and to assess preclinical efficacy of novel therapeutic interventions like gene augmentation, splice modulation or CRISPR/Cas9-based genome editing. Although there are still several challenges to overcome, such as increasing the efficiency of delivering exogenous DNA or other therapeutic molecules, and optimizing outer segment biogenesis, the enormous potential of this system is obvious. In particular, the findings of Parfitt *et al.* concerning the differential splicing of *CEP290* have triggered to further unveil the mysteries of photoreceptor-specific pre-mRNA splicing in the human retina. This will allow us to improve molecular diagnostics, better understand the relationship between splice mutations and the pathophysiological mechanisms underlying IRD, and thereby speed up the development of novel therapeutic interventions for these severe blinding disorders.

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Footnote

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