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Ocular Ciliopathies: Genetic and Mechanistic Insights into Developing Therapies

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Abstract

Developing suitable medicines for genetic diseases requires a detailed understanding of not only the pathways that cause the disease, but also the identification of the genetic components involved in disease manifestation. This article focuses on the complexities associated with ocular ciliopathies – a class of debilitating disorders of the eye caused by ciliary dysfunction. Ciliated cell types have been identified in both the anterior and posterior segments of the eye. Photoreceptors (rods and cones) are the most studied ciliated neurons in the retina, which is located in the posterior eye. The photoreceptors contain a specialized light-sensing outer segment, or cilium. Any defects in the development or maintenance of the outer segment can result in severe retinal ciliopathies, such as retinitis pigmentosa and Leber congenital amaurosis. A role of cilia in the cell types involved in regulating aqueous fluid outflow in the anterior segment of the eye has also been recognized. Defects in these cell types are frequently associated with some forms of glaucoma. Here, we will discuss the significance of understanding the genetic heterogeneity and the pathogenesis of ocular ciliopathies to develop suitable treatment strategies for these blinding disorders.

Keywords

Leber congenital amaurosis; Photoreceptors; genetic diseases; ocular ciliopathies

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CONFLICT OF INTEREST

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CONSENT FOR PUBLICATION

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1. INTRODUCTION

The field of molecular genetics has come a long way since the initial isolation and characterization of DNA by Friedrich Miescher [1]. Significant efforts are now underway in order to confidently recognize genetic mutations and to predict the penetrance and severity of the associated diseases.

With the identification of disease-associated genes and the elucidation of their functions, there has been a large increase in the research endeavors helping to develop molecular therapeutics. Therapies for genetic disorders include delivering the correct (non-mutated) copy of the diseased gene – in case of autosomal recessive diseases – and knocking down the expression of the mutant allele (in case of autosomal dominant diseases) [2]. Recent advances in the genome editing technologies have also allowed for precisely modifying gene sequences within the genome [3].

While the current gene therapeutic approaches to genetic disorders are promising during early treatment stages, their long-term effect diminishes. This is largely due to an incomplete understanding of the disease process and the complex interplay between different pathways and proteins that manifest the disease. Such complexities are evident from the highly heterogenic nature of both the genetic component of the disease and its clinical presentation.

Here we focus on our current understanding of the complex disease processes associated with ciliopathies, which are a class of genetically and clinically heterogeneous disorders that are caused due to the dysfunction of the cilia.

2. CILIA

Cilia are microtubular extensions of the plasma membrane of nearly all cell types. The nucleation of ciliary microtubules occurs at the basal body (also called mother centriole) in the G₀-G₁ phase of the cell cycle. Ciliogenesis is ensued by the polymerization of nine outer doublet microtubules into a structure known as the transition zone (TZ). Further polymerization into singlet microtubules gives rise to the distal axoneme. Depending upon the presence of two inner microtubules, the cilia can either be classified as motile (inner microtubules present; 9+2 array) or primary (nonmotile; no inner microtubules; 9+0 arrangement) [4–7].

Cilia maintain a unique composition of the membrane and soluble proteins. This is made possible by a stringently controlled selection and transport process. The ciliary membrane cargo is trafficked by the Intraflagellar transport (IFT). The IFT machinery consists of macromolecular complexes of IFT proteins, including motor proteins kinesin motor subunits (anterograde transport) and cytoplasmic dynein motor (retrograde movement). The cargo enters the cilia by the activity of the kinesin-driven machinery whereas its exit is mediated by dynein-regulated transport. The retrograde trafficking is further modulated by additional proteins, such as the BBSome (a complex of selected Bardet-Biedl Syndrome-associated proteins) [8–10]. Recent studies have also provided evidence for the ectosome-mediated shedding of some ciliary membrane receptors from the cilia [11–12]. Additional

cilia-associated protein complexes are involved in regulating many diverse processes, including vesicle transport, cargo delivery, and ciliary signaling cascades [13].

Although there is a vast diversity in the composition of the cilia, these protein complexes are united in their efforts to carry out complex ciliary functions. Primary cilia usually function as environmental sensors (mechanosensors, chemosensors or neurosensors). While functioning as sensors, the primary cilia mediate several perceptions, including audio perception (cochlea), olfaction (olfactory cilia), and vision (rods and cones) [5, 14]. Given the involvement of cilia in diverse cellular processes, ciliary dysfunction results in a multitude of disorders that are collectively termed ciliopathies. These include Meckel-Gruber syndrome, Joubert Syndrome, Bardet-Biedl Syndrome, Senior-Loken Syndrome and Usher Syndrome. Details of the ciliary protein complexes and their involvement in human diseases have been discussed in detail elsewhere. [15–20].

In the next sections, we will discuss the involvement of ciliary function in regulating the structures and functions of the eye.

3. RETINA

The retina, situated in the back of the eye, is a specialized tissue that assists in light detection and signaling (Fig. 1A). It consists of multiple layers of neurons involved in the detection and transmission of a light signal in the form of nerve impulses. Among these neurons, the photoreceptors (rods and cones) are the most abundant and commonly studied cell types in the retina. Photoreceptors are highly specialized neurons with distinct compartments: the cell body, the inner segment, and the outer segment (Fig. 1B). The outer segment is a modified cilium, which contains densely packed membranous discs loaded with opsins. The inner segment of photoreceptors contains the protein synthesis and trafficking machinery and other organelles [21–23].

Although both rods and cones share a similar overall arrangement in their different compartments, they vary in their shape, size and light detecting abilities. Rods are highly sensitive and active only in dim light (or starlight) and have long cylindrical structures with the ciliary OS membrane enclosing the membranous discs. The discs are loaded with the photopigment opsin (a G protein-coupled receptor) and other proteins involved in the phototransduction cascade. Cones on the other hand, are relatively shorter with discs in the ciliary OS continuous with the plasma membrane [21]. Additionally, mice have two types of cones: short wavelength (S) cones and medium wavelength (M) cones, which are responsive to respective light intensities. In contrast, the human retina has a third cone subtype, called long wavelength (L) cones [24].

3.1. Photoreceptor Sensory Cilia

The sensory cilium of a photoreceptor can be divided into three main compartments: outer segment containing the axoneme, TZ and basal body (BB) (Fig. 1B). The TZ is connected to the plasma membrane by Y-shaped linker structures [25]. It is approximately 200–500 nm in length and 170 nm in diameter and is proposed to serve as a bottleneck or a gate for the entry of proteins into the OS. While there is a high degree of conservation in overall

morphology and structure-function relationship in cilia from diverse cell types, the photoreceptors develop unique and highly modified sensory cilia: (i) The photoreceptors form an elaborate structure in the outer segments that consists of numerous membranous discs arranged in a coin stack-like fashion. These discs are loaded with the photopigment rhodopsin; (ii) The outer segments undergo periodic shedding of their distal tips. The shed outer segment tips are phagocytosed by the overlying retinal pigment epithelium (RPE), a layer of phagocytic cells that also provides nutrients to the photoreceptors. The loss of the distal tips is compensated by the simultaneous addition of new discs and membranes at the base of the outer segment. Both the shedding and renewal of the outer segment exhibit stringent circadian regulation; (iii) The involvement of the retrograde movement of the cargo mediated by cytoplasmic dynein in photoreceptor cilia is unclear [21, 26–27]. Recent studies though have indicated that the retrograde movement may involve recycling of the IFT proteins rather than of a cargo [26–27]. Thus, disruption of the retrograde machinery may affect photoreceptor ciliary formation and maintenance, which may lead to eventual ciliary dysfunction and photoreceptor degeneration.

The unique features of the photoreceptor cilia suggest that these cell types have developed specific regulatory mechanisms. These involve expression and function of specific proteins or distinct protein-protein interactions. Proteins that localize to distinct compartments in the photoreceptor cilia exist as parts of multiprotein complexes. For example, the TZ-associated proteins RPGR and CEP290 form complexes with each other as well as with other proteins including RPGRIP1, RPGRIP1L, NPHP1, NPHP4, NPHP5, and NPHP8 [21, 28–32]. Distal axonemal proteins RP1 and MAK interact with each other to modulate outer segment stability. A functional balance between MAK and RP1 is thought to be essential for the regulation of ciliary length and proper formation of the ciliary sub compartments. Excess activation of RP1 has been shown to induce ciliary elongation. [33]. Overall, these protein complexes maintain the morphology of the photoreceptor cilia. Interestingly, the majority of the ciliary proteins identified to date are associated with the retinal degenerative disease. [34].

3.2. Anterior Segment Cilia

There are three major fluid-filled chambers in the eye: anterior chamber, which is between the cornea and the iris; posterior chamber, between the iris and the lens and vitreous chamber, which is located between the lens and the retina and is filled with vitreous humor (Fig. 1A). The anterior and the posterior chambers are filled with a fluid called aqueous humor, which supplies nutrients to the cornea and the lens and maintains optimum pressure in the eye, called intraocular pressure (IOP). The IOP is regulated by a balanced inflow and outflow of aqueous humor from the anterior chamber into the blood stream. A major pathway for outflow is via the trabecular meshwork into the Schlemm's canal [35].

Recent studies have identified a novel role of cilia in the trabecular meshwork cells to regulate fluid outflow. It was found that primary cilia in these cells respond to fluid flow and hydrostatic pressure. This function is mediated by OCRL, a gene implicated in Lowe Syndrome. OCRL interacts with a ciliary membrane receptor TRPV4 (transient receptor

potential cation channel subfamily V member 4) and regulates its downstream calcium signaling [36–37].

4. POTENTIAL ROLE OF CILIA IN OCULAR ANGIOGENESIS

Angiogenesis is the sprouting of new blood vessels from pre-existing vessels. Defective angiogenesis is a leading cause of debilitating ocular disorders, such as glaucoma, age-related macular degeneration, retinopathy of prematurity, and diabetic retinopathy. Primary cilia on endothelial cells of blood vessels are involved in sensing blood flow-induced stress as well as detection of soluble moieties/ligands in the blood. Remarkable studies revealed that endothelial cilia are involved in regulating angiogenesis by modulating calcium signaling cascades [38]. A variety of membrane receptor tyrosine kinases act as receptors for their respective ligands to regulate angiogenesis. These include TEK and vascular endothelial growth factor receptors (VEGFRs: VEGFR1 and VEGFR2). [39].

TEK and its ligands ANGPT1 and ANGPT2 have been implicated not only in the development and maintenance of the Schlemm's canal but also in the pathogenesis of glaucoma [40–42]. Given their involvement in regulating endothelial cell function, we asked whether TEK localizes to cilia. Immunostaining of ciliated Human umbilical vein endothelial cells (HUVECs) with an anti-TEK antibody showed that the TEK receptor predominantly localizes to the cilia (Fig. 2A). Cilia were identified using an antibody against the acetylated α -tubulin (ACT-TUB), a ciliary marker. Moreover, we recently found a potential genetic and physical interaction between *TEK* and *CYP1B1* (most frequently mutated gene in primary congenital glaucoma) [43]. We therefore, tested the localization of CYP1B1 with respect to cilia. Our analysis revealed that, in addition to a predicted cytosolic localization, CYP1B1 localizes to the base of the cilium in the HUVECs (Fig. 2B). In a similar assay, we found that VEGFR2, the major receptor implicated in angiogenesis, is also localized predominantly to the cilia in HUVECs (Fig. 2B). These results provide compelling evidence for a potential role of cilia in mediating endothelial receptor signaling cascades mediated by TEK and VEGFR2. Thus, studies should be initiated to understand the involvement of ciliary dysfunction in the pathogenesis of angiogenesis disorders, such as Diabetic retinopathy and wet age-related macular degeneration.

5. COMPLEXITIES OF OCULAR CILIOPATHIES

Ocular ciliopathies are a subclass of ciliopathies that occur due to ciliary dysfunction in the cell types involved in regulating the structure and function of the eye. These ciliopathies present either as non-syndromic forms or as part of syndromic diseases. There is immense genetic heterogeneity in ciliopathies. There are about 180 known ciliopathy-associated genes. In some cases, mutations in a gene can cause multiple distinct ciliopathies. Moreover, depending upon the pathogenic potential of the alleles, the single gene can be linked to multiple phenotypes of variable strength and penetrance. In general, predicted missense mutations in ciliopathy genes, are thought to result either in a gain or reduction of function as opposed to nonsense mutations that may result in a loss of function. For example, mutations in *IFT172* and *IFT43* are associated with skeletal defects as well as retinal degeneration and BBS [44–45]. Mutations in *CEP290* are also associated with a spectrum of

disorders, including Leber congenital amaurosis (LCA), Senior-Loken syndrome, BBS, Joubert Syndrome and Meckel-Gruber syndrome [16, 34, 46]. The contribution of specific types of allelic variants on the function of these proteins is still unclear; however, they are thought to affect distinct functions of the ciliary proteins.

The genes that encode multiple isoforms are also associated with heterogenic clinical manifestations. This is because the different isoforms may play overlapping as well as distinct roles in the cilia. An alternatively spliced isoform of BBS3 was found to be specifically required for photoreceptor function whereas mutations in another BBS3 isoform are associated with BBS [47].

In addition to single gene disorders, multiple gene-gene interactions have been proposed to either mediate or modulate the disease phenotype. Several ciliopathy-associated proteins genetically interact with each other and form multiprotein complexes in the cilia. The composition and function of these complexes are altered due to mutations in single or multiple component proteins. A mutated protein in a complex may either result in a different composition of the component proteins or disrupt the whole complex. In another scenario, one mutated protein in the complex may reduce the activity of the whole complex while two or more mutated components of the complex may perturb the formation or the function of the complex. It is the latter scenario that is difficult to detect. Examination of genetic interactions and their perturbation due to mutations has provided new knowledge of their role in the manifestation of pleiotropic ciliary dysfunction, including retinal ciliopathies. For example, the effect of the loss of *Bbs4* or *Bbs6* genes in mice is either exacerbated or reduced by a concomitant loss of *Cep290* [48–49]. In another study, a heterozygous *Cep290* allele exacerbated the severity of retinal degeneration of the *Rpgt*^{ko} mouse [50]. Similar dosage-dependent genetic interactions between the *Ahi1* and *Nphp1* genes also increased the rate of progression of photoreceptor degeneration in mice and humans [51]. A missense mutation in the Joubert Syndrome-associated gene *RPGRIP1L* gene was found to be associated with the manifestation of retinal degeneration in pleiotropic ciliopathies. [30].

Although retinal degeneration is commonly detected in the ciliopathies, the effect of ciliary dysfunction distinctly affects the rods and cones. Some retinal ciliopathies are associated with relatively earlier rod dysfunction and degeneration. These diseases are broadly classified as retinitis pigmentosa (RP) [52–53]. RP is a clinically and genetically heterogeneous group of photoreceptor degenerative diseases affecting 1 in 3000–5000 people worldwide. It manifests as night blindness (rod dysfunction) usually in the second decade of life, followed by progressive loss of daytime vision (cone dysfunction) and legal blindness. The clinical manifestation of RP varies with the age of onset and the rate of progression of photoreceptor degeneration [52–53]. At the genetic level, RP is inherited in autosomal dominant, autosomal recessive, and X-linked disease. Some forms of mitochondrial inheritance have also been reported [54–55]. Moreover, mutations in ~200 genes have been reported to be associated with RP, making it one of the highly genetically heterogeneous forms of inherited disorders. Mutations in several genes, including Rhodopsin, *PRPF31*, *RP1*, *RP2*, *RPGR*, and *TOPORS* have been implicated in RP due to ciliary dysfunction [13, 56–60].

Cone-rod degeneration is a form of photoreceptor degeneration that manifests as early impairment of color vision due to cone dysfunction followed by night blindness (rod dysfunction). Mutations in ciliary transition zone protein encoding genes, including *RPGR*, *RP2*, *TTL5* (tubulin tyrosine ligase-like 5), and *RPGRIP1* are also associated with some forms of conerod degeneration [61–63]. It is intriguing that mutations in the same gene can cause RP as well as cone-rod dysfunction. These observations suggest either that ciliary proteins play distinct roles in rods and cones or that ciliary dysfunction is differentially regulated among these neurons.

Detailed analysis of the rodor cone-dominant mouse models and canine models of retinal ciliopathies has provided new knowledge on the distinct responses of the rods and cones to ciliary dysfunction [64–66]. Although the causative gene is expressed in both cell types, rods and cones can distinctly regulate the onset of the gene expression and/or the function of the proteins. Such differences are implicated in the differential phenotypic presentation of rods and cones. RNAseq analyses of animal models have also shown that the rods and cones respond to the disease-causing mutation by differentially regulating specific intracellular pathways [65]. Furthermore, a recent study showed that the ablation of the *Rp2* gene, mutations in the human counterpart of which are associated with some forms of X-linked retinitis pigmentosa and cone-rod degeneration, resulted in abnormal extension of the cone sensory cilium and the outer segment [59, 67]. This effect was not observed in rod photoreceptors. In another study, loss of *RPGR* in a rod or cone dominant retina produces distinct effects on photoreceptor morphology and function [65]. Consistently, clinical studies have shown that the mutations in *RPGR* and *RP2* may manifest in typical retinitis pigmentosa or a cone-rod degeneration phenotype [63, 68–69]. A previous study using zebrafish as a platform to assess the pathogenic potential of human *RP2* mutations showed that some of them are partly functional in one cell type but not in the other [70].

5.1. Anterior Segment Ciliopathies

Given that ciliopathies in general and retinal ciliopathies in particular, are genetically heterogeneous diseases, such genetic complexity is also predicted in anterior segment ciliopathies. Cilia in the cells of the anterior segment are implicated in IOP regulation and modulating inositol phosphatase levels in the trabecular meshwork. The IOP serves as an important risk factor for developing an irreversible blindness disorder called glaucoma [23, 71].

Mild alterations in the ciliary levels of inositol phosphatases either directly due to mutations in the phosphatase gene or in one of the regulators of the phosphatases can modulate the underlying ciliary pathology of the trabecular meshwork [72]. In addition, the trabecular meshwork cells exhibit a complex network of actin filaments that are thought to provide a filter-like structure. The formation of the actin network is inversely associated with cilia formation in several cell types, including the photoreceptors. Hence, an overlap of actin-related defects with ciliary dysfunction in the trabecular meshwork also cannot be ruled out.

6. OUTLOOK

As ciliopathies are usually inherited in an autosomal recessive manner, gene replacement therapy is a viable option for these diseases. However, additional studies should be carried out to identify genetic modulators of disease expression. These studies would involve large scale data collection (both clinical and genetic), specifically for identifying rare alleles and their interactions.

The molecular insights obtained from studying the mechanisms of ocular ciliopathies (using information of the genes involved in the disease) have provided new avenues for designing small molecule-based treatments. A deep intronic mutation in *CEP290* (c.2991+1655A>G) is commonly observed in LCA [46]. This mutation disrupts normal splicing by creating a cryptic splice site between exons 26 and 27. Antisense oligonucleotides (AONs), which are small RNA molecules that bind to target mRNA and interfere with splicing, were used to restore normal splicing in patient fibroblasts and in a humanized *Cep290*-mutant mouse [73–74]. In addition, translational read-through drugs, such as aminoglycoside antibiotics have been demonstrated to be highly effective for restoring full-length protein translation from mRNAs with premature termination codons. This approach was used to restore RP2 expression and function in a patient's induced pluripotent stem cell-derived RPE cells carrying RP2 R120X mutation [75].

Gene replacement therapy is at the forefront of developing longer-lasting medicines for inherited disorders. Adeno-associated viral (AAV) vectors provide a relatively safer vehicle to deliver mutation-free cDNAs into the diseased tissues [76]. Recent FDA-approval of RPE65 gene therapy to treat a form of LCA has infused confidence in the field. AAV-mediated delivery of ciliary disease genes, including *RPGR*, *RP2*, and *BBS4* has been shown to be effective in animal models [77–79]. However, there is a pitfall associated with conventional AAV-mediated gene delivery. The AAV virus genome has a packaging limit of 4.5–5 kb [76]. Thus, it is not amenable for delivery of large genes, such as *CEP290*. Although alternative AAV strategies such as dual or triple AAV vectors have been used, their *in vivo* efficacy remains to be determined [80]. To overcome the AAV load limit roadblock, shorter yet partly functional forms of large genes were used. The knowledge of the function of the distinct domains of the CEP290 protein in cilia targeting and function led to the generation of miniCEP290s that exhibited partial improvement in photoreceptor structure and function of *Cep290*^{rd16} mice [81]. These miniCEP290s can be used both *in vitro* in mutant mouse fibroblasts and *in vivo* using AAV-mediated gene delivery strategy. This strategy also allows for a mutation-independent approach for *CEP290*-disease.

Majority of glaucoma cases manifest higher intraocular pressure. Some glaucoma medications currently prescribed to reduce the intraocular pressure include prostaglandin analogs, β -blockers, α 2-agonists, carbonic anhydrase inhibitors, as well as combination formulations of these drugs. Sometimes surgical interventions are needed to reduce the pressure [82]. However, these strategies are associated with side effects and are not long-term solutions. Using the knowledge of ciliary dysfunction, a TRPV4 agonist in the trabecular meshwork reduced the IOP. This provides a promising approach to further delay the progression of glaucoma [36]. Regulation of angiogenesis using antiVEGF antibodies,

TEK modulators or Angiopoietin agonists can assist in controlling the pathogenesis of not only macular degeneration and diabetic retinopathy but also congenital glaucoma. Since VEGFR2 and TEK localize to cilia, disrupting or modulating the pathways that specifically target these receptors to the cilia could also be developed as a therapeutic strategy for associated diseases. Given the involvement of ciliary signaling in endothelial cells, future studies can focus on designing modulators to fine-tune the availability of ciliary membrane receptors to mediate the specific signaling cascades.

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LIST OF ABBREVIATIONS

ACT-TUB	Acetylated α -tubulin
AHI1	Abelson Helper Integration Site 1
BBS	Bardet-Biedl Syndrome
CEP290	centrosomal protein 290 kDa
CYP1B1	cytochrome p450–1B1
HUVECs	Human umbilical vein endothelial cells
IOP	Intraocular pressure
LCA	Leber congenital amaurosis
MAK	male germ cell associated kinase
NPHP	Nephronophthisis
RP2	retinitis pigmentosa 2
RPGR	retinitis pigmentosa GTPase regulator
RPGRIP1L	RPGR-interacting protein 1-Like
TEK	Tyrosine kinase, endothelial
TRPV4	Transient receptor potential subfamily V member 4
VEGF	vascular endothelial growth receptor

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Fig. (1).

A schematic diagram of the side-view of the eye ball (A) and the photoreceptors (B) is depicted. BB: basal bodies; ER: endoplasmic reticulum; G: Golgi; M: mitochondria; TZ: transition zone.

Fig. (2).

Ciliary staining of endothelial receptors: **A:** Ciliated HUVECs were immunostained with TEK (green; Proteintech) or CYP1B1 (green; Proteintech) and acetylated- α -tubulin (ACT-TUB; cilia marker; red; Sigma) antibodies. Merge image shows co-localization of the red and the green signals. Arrows indicate cilia in the TEK-stained panel whereas in the CYP1B1-stained panel, arrows indicate the CYP1B1-immunoreactive signal at the base of cilia. **B:** Ciliated HUVECs were stained with VEGFR2 (red; Sigma) or ARL13B (ADP-

ribosylation Factor-like 13B; ciliary marker; green; Proteintech). Arrows indicate ciliary staining of VEGFR2.

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