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Olfactory loss and dysfunction in ciliopathies: Molecular mechanisms and potential therapies

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Abstract

Background—Ciliopathies are a class of inherited pleiotropic genetic disorders in which alterations in cilia assembly, maintenance, and/or function exhibit penetrance in the multiple organ systems. Olfactory dysfunction is one such clinical manifestation that has been shown in both patients and model organisms. Existing therapies for ciliopathies are limited to the treatment or management of symptoms. The last decade has seen an increase in potential curative therapeutic options including small molecules and biologics. Recent work in multiciliated olfactory sensory neurons has demonstrated the capacity of targeted gene therapy to restore ciliation in terminally differentiated cells and rescue olfactory function. This review will discuss the current understanding of the penetrance of ciliopathies in the olfactory system. Importantly, it will highlight both pharmacological and biological approaches, and their potential therapeutic value in the olfactory system and other ciliated tissues.

Methods—We undertook a structured and comprehensive search of peer-reviewed research literature encompassing *in vitro, in vivo*, model organism, and clinical studies. From these publications, we describe the olfactory system, and discuss the penetrance of ciliopathies and impact of cilia loss on olfactory function. In addition, we outlined the developing therapies for ciliopathies across different organ and cell culture systems, and discussed their potential therapeutic application to the mammalian olfactory system.

Results—One-hundred sixty-one manuscripts were included in the review, centering on the understanding of olfactory penetrance of ciliopathies, and discussing the potential therapeutic options for ciliopathies in the context of the mammalian olfactory system. Forty-four manuscripts were used to generate a table listing the known congenital causes of olfactory dysfunction, with the first ten listed are linked to ciliopathies. Twenty-three manuscripts were used to outline the potential of small molecules for the olfactory system. Emphasis was placed on HDAC6 inhibitors and lithium, both of which were shown to stabilize microtubule structures, contributing to ciliogenesis and cilia lengthening. Seventy-five manuscripts were used to describe gene therapy and gene therapeutic strategies. Included were the implementation of adenoviral, adeno-associated

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

virus (AAV), and lentiviral vectors to treat ciliopathies across different organ systems and application toward the olfactory system. Thus far, adenoviral and AAV-meditated ciliary restoration demonstrated successful proof-of-principle preclinical studies. In addition, gene editing, *ex vivo* gene therapy, and transplantation could serve as alternative therapeutic and long-term approaches. But for all approaches, additional assessment of vector immunogenicity, specificity, and efficacy need further investigation. Currently, ciliopathy treatments are limited to symptomatic management with no curative options. However, the accessibility and amenability of the olfactory system to treatment would facilitate development and advancement of a viable therapy.

Conclusions—The findings of this review highlight the contribution of ciliopathies to a growing list of congenial olfactory dysfunctions. Promising results from other organ systems imply the feasibility of biologics, with results from gene therapies proving to be a viable therapeutic option for ciliopathies and olfactory dysfunction.

Keywords

olfactory dysfunction; ciliopathy; olfactory epithelium; gene therapy; small molecule

INTRODUCTION

Cilia are organelles present on the surface of cells in a wide variety of organisms from unicellular flagellates and round worms to almost all cell types in vertebrates. Cilia have a diverse set of functions, including propulsion and fluid movement for motile cilia as well as cellular signaling and detection of external stimuli such as growth factors, sound, light, and odors for non-motile cilia [1–3]. While our understanding into the diverse biological functions of cilia is continually increasing, so is our comprehension for their role in disease. Ciliopathies are a class of pleiotropic congenital human diseases with phenotypes that exhibit variable penetrance depending on the tissue, the gene and the type of mutation, as well as an individual's genetic background [4,5]. Clinical manifestations of ciliopathies can occur during development and throughout adulthood and may result in a variety of deficits including polydactyly, obesity, hypogonadism, renal dysplasia, infertility, situs inversus, respiratory complications, liver fibrosis, retinal dystrophy, and anosmia [5].

The olfactory epithelium (OE) is a pseudo-stratified epithelium that lines the nasal turbinates and septum and is comprised of multiple cell types including immature and mature olfactory sensory neurons (OSNs), supporting sustentacular cells, and two populations of stem cells known as globose basal cells and horizontal basal cells (HBCs) (Figure 1.). Within the OE, developing neurons possess primary cilia during embryogenesis [6,7], however their function is currently unknown. In addition, recent work has shown that HBCs possess primary cilia that are involved in regulating regeneration of the OE after injury [8]. Unlike developing neurons and HBCs, mature OSNs are multiciliated bipolar neurons that project from their dendritic knob approximately 20-35 cilia, extending up to ~100 μm in length, into the olfactory lumen and cover the surface of the OE [9]. Located within these cilia are odorant receptors, which are seven-transmembrane G-protein coupled receptors (GPCRs) that bind odor molecules within the nasal cavity. GPCRs are often pharmacological targets for therapies, due in part to their involvement in various diseases [10–13]. In addition to

odorant receptors, olfactory cilia also contain signal transduction machinery required for odor detection including the heterotrimeric G-proteins, adenylyl cyclase III (ACIII; EC 4.6.1.1), cyclic nucleotide gated channels, and calcium activated chloride channels. Extending from basal bodies that are organized in a ring-like fashion around the dendritic knob, OSN cilia have a transition zone, proximal segment, and a long distal segment. The proximal segment (~1-2 μm) of cilia is made up of microtubule doublets, while the distal segment (on average, 50 μm in length) is made up microtubule singlets [9]. Much like primary cilia of other cells, OSN cilia utilize anterograde and retrograde intraflagellar transport for protein movement along the ciliary axoneme [9]. Importantly, mutations in genes associated with the structural integrity or function of cilia can lead to olfactory dysfunction.

Alterations in olfactory function can range from hyposmia (reduction of olfactory function) and anosmia (loss of olfactory function), to hyperosmia (increased olfactory acuity) and dysosmia (distorted odor perception). In patients, olfactory dysfunction can impact their personal safety and reduce their quality of life [14–16]. Olfactory dysfunction is attributed to multiple causes, which include post-viral infection, head trauma, inflammation or chronic sinusitis [17]. In addition, there is a growing list of olfactory dysfunctions resulting from congenital causes, which includes ciliopathies (Table 1). The degree of penetrance of ciliopathies within the olfactory system is not well understood, nor are the associated mechanisms of action and disease phenotypes. However, over approximately the last 10 years, research in both ciliopathy patient populations and murine models has gradually started examining the occurrence and mechanisms of olfactory dysfunction in ciliopathies. Individuals with Bardet-Biedl Syndrome (BBS) as well as Leber congenital amaurosis (LCA) exhibit abnormal or anosmic scores on the B-SIT olfactory function test [18,19]. In murine ciliopathy models such as the Oak Ridge Polycystic Kidney (ORPK) mouse, as well as in knockouts of BBS1, BBS4, or BBS8, ciliation was either significantly reduced or absent from OSNs. In addition, these mice exhibit loss of olfactory function and significantly reduced olfactory bulb activity, while BBS8 knockout mice also show OSN axon mistargeting in the olfactory bulb [18,20,21]. In addition, loss of BBS1 in OSNs results in mistrafficking of core BBS complex proteins to cilia, causing a reduction in cilia length and number as well as reduced olfactory bulb activity [22]. Interestingly, in *rd16* mice, which possess a genetic deletion of *Cep290*, cilia are still present on the surface of the OE. However, G proteins essential for olfactory signal transduction do not traffic into cilia and therefore *rd16* mice are functionally anosmic [19]. These findings suggest that ciliopathies have varying degrees of penetrance in the OE and that the mechanisms underlying loss of olfactory function may vary. This may also be true for cells in the OE that elaborate primary cilia. Though primary cilia have been identified on developing OSNs [6,7], their function as well as their potential role in ciliopathy-related anosmia has yet to be determined. Recent work has shown that loss of primary cilia on HBCs, due to deletion of either *Ift88* or *Arl13b*, two ciliopathy related genes, impairs the ability of HBCs to regenerate the OE after injury [8]. This work suggests that beyond OSNs, olfactory ciliopathies exhibit penetrance in stem cells of the OE and that impaired neurogenesis may also be a potential mechanism for congenital anosmia.

Despite our incomplete understanding of the penetrance of cilia dysfunction within the olfactory system, both small molecule and gene therapies offer potential to treat olfactory ciliopathies. In this context, we address the current and future challenges for treating patients with olfactory dysfunction.

SMALL MOLECULE THERAPEUTIC STRATEGIES

While no small molecule therapies have been developed specifically for ciliopathy-induced olfactory dysfunction, the use of pharmaceuticals for treatment of ciliopathies in other organ systems, particularly for BBS and polycystic kidney disease (PKD), have shown progress. In BBS, cilia dysfunction leads to retinal degeneration [23–28] via protein accumulation in the inner segment of photoreceptors, stress of the endoplasmic reticulum, and the subsequent activation of the proapoptotic unfolded protein response [29]. Interestingly, in a murine model of BBS12, Mockel *et al* [29] showed that administration of a combination of valproic acid, guanabenz, and a specific caspase-12 inhibitor reduces apoptosis and preserves light detection in mutant animals. In PKD, extensive research and clinical trials of pharmacological therapies are ongoing. These studies frequently use pharmacological inhibitors to target cilia-associated cell proliferation signaling pathways such as mTOR (mammalian target of rapamycin), cAMP (cyclic adenosine monophosphate), and EGFR (epidermal growth factor receptor) [30–34]. Typically, reductions in kidney or liver volume were observed but kidney function was not completely restored [34–36]. More recently it has been demonstrated that cell proliferation in renal tubules was not sufficient to induce cyst formation after cilia disruption [37] and that cyst growth is regulated by the primary cilium, independent of cell proliferation signaling [38]. Other studies suggest that alterations in the length and/or stability of the primary cilium may be an important direct regulator of PKD pathogenesis [39–41].

Recent work has shown that histone deacetylase 6 (HDAC6; EC 3.5.1.98) is a major regulator of cilia stability and disassembly, and could be an important therapeutic target for ciliopathies [42]. Signaling proteins such as calmodulin and β-catenin as well as the ciliary protein nphp2/inversin interact with Aurora A, which phosphorylates and activates HDAC6, leading to deacetylation of α-tubulin and cortactin [43–46]. Deacetylation of a-tubulin leads to disassembly of primary cilia, while deacetylation of cortactin promotes interaction with filamentous actin (F-actin), ultimately leading to ciliary resorption [46]. Interestingly, calmodulin is involved in adaptation during olfactory signal transduction. It would be intriguing to examine whether calmodulin has a similar role in regulating ciliary disassembly via HDAC6 in the olfactory system and whether HDAC6 inhibitors could be used as a potential therapeutic option for olfactory dysfunction. In primary cilia, an HDAC6-specific inhibitor tubastatin A, has been shown to increase acetylated α-tubulin and restore ciliation and cilium-dependent processes [47]. Moreover, non-selective HDAC inhibitors including vorinostat, an FDA approved cancer drug, have also demonstrated increased ciliogenesis of primary cilia [42,47]. However, tubulin targeting chemotherapeutic cancer drugs such as paclitaxel (taxol) have shown negative effects on the OE, leading to severe lesions [48]. In zebrafish, characteristic ciliopathy phenotypes were observed upon reduction of BBSome-interacting protein of 10 kDa (BBIP10), a protein required for microtubule acetylation and assembly of the primary cilia [49]. In BBIP10-depleted cells, application of tubacin, an

HDAC6 inhibitor, restored microtubule acetylation and ciliary assembly providing evidence that BBIP10 regulates tubulin acetylation by inhibiting HDAC6 [49]. As the BBSome is an important regulator of cilia function in the olfactory system [9], this work is suggestive that HDAC6 inhibitors may be a potential therapeutic target for olfactory ciliopathies.

Interestingly, lithium, which has been used to treat neurological disorders, also promotes tubulin acetylation and elongation of cilia [50]. Specifically, brain sections of mice chronically fed lithium carbonate demonstrate primary cilia elongation within the dorsal striatum and nucleus accumbens [51], while in cultured cells lithium chloride has also been shown to elongate cilia [50–52]. Lithium Chloride promotes acetylation of α-tubulin through the activation of α-tubulin N-acetyltransferase 1 (α-TAT1; EC 2.3.1.108) and inhibition of glycogen synthase kinase 3β (GSK3β; EC 2.7.11.26), resulting in elongation of primary cilia [50]. While lithium may increase cilia length it is currently unknown whether this is sufficient for restoring cilia function.

The use of small molecule therapy for treating olfactory dysfunction is enticing. However, extensive studies on their effects *in vivo* and efficacy in the context of the olfactory system remains unexplored. It also remains unclear whether restoration of cilia morphology is sufficient to reconstitute ciliary function, especially in multiciliated OSNs. In ciliopathies such as BBS, ciliary elongation and/or microtubule stabilization may be inadequate to restore the function of the missing BBS protein or the BBSome complex, which is critical for protein trafficking. Importantly, small molecule therapies are generally not curative treatments and other therapeutic approaches for restoration of olfactory function, such as gene therapy, should also be considered.

GENE THERAPEUTIC STRATEGIES

The first clinical trials of gene therapy to human patients were performed between 1989 and 2000 using retroviral and adenoviral-mediated transduction. Since then, the field has advanced dramatically with modifications of those original vectors and the development of adeno-associated viral (AAV) vectors, which demonstrated tissue specificity and lower immunogenic response, and the expansion of non-viral mediated strategies [53–55]. Clinical trial successes have been shown in multiple diseases, such as sickle cell anemia, hemophilia, cystic fibrosis, and retinal degeneration. More recently, a major breakthrough occurred in the field with the use of gene therapy for CAR-T cells and cancer immunotherapies. During the time of this review, the United States Food and Drug Association approved tisagenlecleucel (Kymriah), a cell-based gene therapy to treat acute lymphoblastic leukemia. To date there are more than 1,200 early stage and active clinical trials that uses gene therapeutic approaches to address multiple diseases (http://www.ClinicalTrials.gov; September 30, 2017; Keywords: Gene therapy). Regardless of the application, the strength of gene therapies is the potential for curative outcomes, especially monogenic diseases such as ciliopathies.

Fundamentally, the strategy of gene therapy is to deliver genetic material that would either replace or correct the disease-causing genetic mutation. The therapeutic gene is packaged in a viral vector, which then infects and is transduced in a host or target cell. The administration can be performed either *in vivo*, where the patient or animal is directly treated

with vector, or *ex vivo*, where cells are collected from the host, transduced, and then transplanted back into the host. In the following section, we will focus on the various vector-mediated gene therapies, which have demonstrated successes in ciliopathy models and the capacity of treating ciliopathies affecting the olfactory system. Much like small molecule treatment, the accessibility of the olfactory system makes it conducive to gene therapeutic delivery. Several studies have demonstrated the receptiveness of the OE to transduction by multiple kinds of viral vectors, generating confidence in these approaches.

Recombinant Adenoviral-mediated Therapy

Adenoviruses are 90-100 nm non-enveloped icosahedral viruses with 57 serotypes. As a vector, adenoviral serotype 5 has been used for the majority of gene therapeutic studies due in part to its ability to efficiently infect multiple cell types, generate high titer preparations, and 3.0-8.0 kb insertional size in its ~36kb linear dsDNA genome. Early adoption of adenoviral vectors for gene therapies were hindered by the strong immunogenic responses from both animal models and human clinical trials [56], resulting in cytokine induction and activation of effector leukocytes [57]. Despite the immunogenic response, their insertion capacity and ease of production make adenovirus ideal for pre-clinical and ongoing gene therapeutic studies with efforts to reduce the immunogenic responses by capsid modifications [56,57].

Outside of the olfactory system, adenoviral-mediated strategies have been applied in ciliopathy models, particularly PKD. *In vitro* reintroduction of wildtype PKD2 (polycystin-2, TRPP2) restored partial function from renal cells isolated from PKD2 knockout mouse model [58]. *In vivo* and in vitro, postnatal PKD1 knockout mice demonstrated slower renal cyst growth 10 days following treatment with adenovirus containing the gene encoding for NGAL (neutrophil gelatinase associated lipocalin), which inhibits proliferation and increases apoptosis [59]. In an alternative strategy, *in vivo* administration of adenovirus encoding for a shRNA for the RAGE (receptor for advanced glycation end products) protein demonstrated slower cyst growth and improved renal function in a mouse model of autosomal dominant polycystic kidney disease (ADPKD) [60].

Within the olfactory field, several studies have demonstrated that the OE and particularly OSNs are amenable to adenoviral-mediated transduction following intranasal delivery [9,21,22,61–69]. Despite earlier reports of infiltration into the olfactory bulb [57,70], infection and transduction was highly restricted to the nasal cavity and OSNs axons [71,72]. Different studies have shown that intranasal delivery of adenovirus infects approximately 15-25% of OSNs, with viral gene expression peaking at 10 days post infection [22,61]. Longevity studies have observed the persistence of gene products to up to 21 days post infection and as long as 90 days post infection; however, there is a dramatic drop in expression levels 30 days post infection [61,72]. Together, the accessibility for non-invasive delivery and the amenability of cells in the OE to viral transduction is an important consideration for gene therapy.

As a tool, adenoviral-mediated expression of fluorescently-tagged proteins has allowed for a greater understanding of the organization and structure of OSN cilia, which includes the detailed morphology, regional divisions, and the dynamics of intraflagellar transport (IFT)

protein trafficking [9]. Important as a potential therapy, the use of the adenoviral vectors is able to restore wildtype olfactory function in ciliopathy and other null mouse models [21,22,64]. First demonstrated in an OMP (olfactory marker protein) knockout mouse model, adenoviral-mediated gene replacement of the OMP gene restored normal odor response kinetics in infected OSNs [64]. The capacity of adenoviral-mediated restoration was bolstered in the ORPK ciliopathy mouse model, which possesses a hypomorphic mutation in *Ift88*. Intranasal delivery of wildtype *Ift88* restored ciliation on OSNs and rescued olfactory function [21]. The success of adenoviral-mediated rescue was recapitulated in a patient-relevant BBS1 knockout mouse model of BBS, where ectopic expression of wildtype BBS1 was capable of restoring ciliary morphology, ciliary IFT protein trafficking, and olfactory function *in vivo* [22]. From the same study, adenoviral exposure and wildtype BBS1 overexpression did not exhibit changes in macrophage infiltration nor apoptosis. Suggesting the delivery and dosage did not induce an immunogenic response nor toxic effects. Together, these studies were the first to demonstrate restoration of ciliation and tissue function in a ciliopathy mouse model. More importantly, these studies provide the preclinical proof-of-concept highlighting the capacity of adenoviral-mediated gene therapy to treat olfactory dysfunctions.

Adeno-associated Viral-mediated Therapy

In the field of gene therapy, safety concerns over adenovirus has increased efforts using adeno-associated viral (AAV) vectors. This focus is due to AAV's natural lack of pathogenicity and limited immunogenic response. Over the years, continual modification and expansion of the AAV serotype library resulted in serotype-dependent cellular specificity and distinct expression levels, onset kinetics, and viral genome copy numbers [73]. To date, there are 12 different serotypes of AAV with different AAV pseudotypes, where the genome from one serotype is packaged within the capsid of a different serotype. AAVs are 20 nm non-enveloped capsid vectors with a ~4.7 kb ssDNA genome. Unlike adenoviral vectors, the insertional capacity of AAV is relatively small and can only accommodate ~2.5 kb of genetic material. Following infection of the host cell, the single stranded AAV genome is processed into dsDNA and undergoes concatemerization, increasing episomal stabilization within the host cell's nucleus. Although the AAV genome remains largely episomal, some AAVs demonstrate the capacity of integrating in the host's genome. Specifically, integration occurs in the long arm of chromosome 19, on site called AAVS1 [74].

AAV-mediated gene therapies have demonstrated preclinical and clinical successes in multiple diseases including hemophilia, cystic fibrosis, and LCA. With regards to ciliopathies, the retina is one tissue that has seen significant successes. LCA is an inherited disease which can result from mutation of cilia genes. It affects retinal pigmented cells and results in severe visual loss shortly after birth. AAV-mediated therapies showed some success in RPE65-null mutations of LCA. In these studies, young and adult RPE65-null canine models exhibited functional and structural recovery of the retina [75–77]. Where 3 years following a single dosage of AAV2/2, AAV2/1, or AAV2/5 serotypes the canine models maintained rod and cone vision [78]. With the success of canine studies, clinical trials using AAV2 containing wildtype *Rpe65* gene to young patients proceeded with

patients also demonstrating success with visual improvement [79]. However, follow up studies show limited long-term effects with patients experiencing continual retinal degeneration despite earlier visual improvement [80,81].

In regards to other ciliopathies, similar successes were observed in the BBS4 knockout and BBS1(M390R) knock-in mouse models of BBS, where retinal degeneration is one of the symptoms [82,83]. Subretinal administration of self-complementary AAV5 containing the wildtype *Bbs4* ciliary gene into BBS4-null mice restored rhodopsin localization, returned normal retinal morphology, and improved electroretinogram recordings [82]. Comparable restorations were observed in BBS1(M390R) knockin mice treated with AAV2/5 containing wildtype *Bbs1*, but did not prevent long-term retinal degeneration [83]. In the nasal cavity, the use of AAV-mediated transduction has been effectively observed in both olfactory and respiratory epithelia exhibiting stable expression up to 9 months in respiratory cells, while also demonstrating the capacity of reinfection [84–86]. More recently, intranasal delivery of AAV9 containing wildtype *Bbs1* restored OSN cilia and olfactory function in a BBS1 knockout mouse model of BBS, recapitulating the results from the adenoviral-mediated therapy [22]. *In vivo* imaging of the infection showed restriction of the treatment to the nasal cavity, and the restoration of odorant detection as recorded by electro-olfactogram 3 weeks post infection. These observations, demonstrate the amenability of the olfactory system and OSNs to AAV-mediated gene therapy. However, more studies are required to examine the efficacy of the strategy for other ciliopathies.

Retroviral-mediated Therapy

Early in the development of gene therapies, retrovirus was the preferred method of gene transfer. The advantage of retrovirus is their ability to stably incorporate into the host's genome, which would allow for long-term and stable gene expression. Retroviruses, such as gammaretroviral and lentiviral vectors, are 100 nm enveloped particles that contain two ssRNAs between 7.0-10.0 kb, with an insertion capacity of 5.0-10.0 kb. Following infection, dsDNA is generated by reverse transcription and integration into the host cell's genome. Lentiviruses differ from other retroviruses by having the pre-integration complex transported to the nucleus, allowing for genetic integration in dividing and non-dividing cells. Use of the retroviral-mediated therapies have demonstrated successes in proof-of-principle pre-clinical studies. Recently in isolated human fibroblasts from CEP290-null LCA patients, lentiviral transduction of the wildtype *Cep290* cilia gene restored primary cilia formation [87]. In studies examining DNAI1-null primary ciliary dyskinesia (PCD), successful lentiviral transduction of the *Dnai1* gene restored ciliary beating in mouse and human respiratory cell cultures [88,89]. Implementation of the lentiviral-mediated therapies to treat olfactory dysfunctions has not been directly attempted. However, cells of the OE demonstrate the capacity of lentiviral transduction both *in vivo* and *ex vivo* isolated OSN cultures [90–92]. The potential use of lentivirus as a gene therapeutic vector is further supported by the repetitive administration of lentivirus without the induction of immune response [93]. In order to gain a better understanding of the capacity of retroviral-mediated treatment of olfactory dysfunctions in ciliopathies, additional studies are required.

Gene Editing Therapy

Despite the success of the gene replacement therapies across multiple diseases, the approach is limited by the longevity of therapeutic gene expression. Both adenoviral and AAV-mediated transductions are dependent on the existence and stability of the episomal genetic material. The issue of longevity is partially addressed by lentiviral transduction, where the therapeutic gene is incorporated into the host cell's genome. However, the insertion sites are nonspecific and demonstrate a risk of insertional oncogenesis or mutagenesis [94,95]. To date, there are three prominent gene editing approaches available: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system [96,97]. ZFNs are artificial restriction enzymes generated by fusing a zinc-finger DNA-binding domain to cleavage domain, which can be engineered to a specific sequence. ZFNs has been shown to efficiently correct the DF508 mutation in the *Ctfr* gene in isolated tracheal cultures from cystic fibrosis patients [98]. More importantly, the correction of the *Ctfr* gene could be performed on isolated pluripotent stem cells and was retained following differentiation [99]. Similar to ZFNs, TALEN-mediated gene editing uses restriction enzymes engineered to cut specific DNA sequences by the fusion a TAL effector DNA-binding domain to a cleavage domain. Application of TALENs as a potential therapeutic agent in ciliopathies has been limited, but correction of the *Dnah11* gene and restoration of the ciliary beating was demonstrated in isolated respiratory cells from PCD patients [100].

In recent years, the CRISPR-Cas9 system has become the primary method for gene editing due to its ease of use and higher specificity. Derived from the prokaryotic immune system, the CRISPR-Cas9 system utilizes a Cas9 nuclease and small guide RNAs to target and alter segments of the target gene. Applications of CRISPR-Cas9-mediated genome editing for treating ciliopathies are still premature, but encouraging results from correcting the IVS26 mutation of the *Cep290* gene of LCA *in vitro* and demonstrating the capacity of CRISPR-Cas9-mediated genome editing in the mouse retina suggest the feasibility of future treatments [101]. Utilization of gene editing within the olfactory field has been limited to use as a tool for generating mutant models. At the time of this review, CRISPR-cas9 gene editing has found success in invertebrate animal models to examine olfactory-guided behaviors and development of the olfactory systems [102–105]. Additional studies are required to determine the feasibility and efficacy of the gene editing in the mammalian olfactory system, but could serve as an alternative long-term solution for treating olfactory dysfunctions.

Ex vivo Gene Therapy

While *in vivo* gene therapy remains the primary methodology for addressing many genetic diseases, the approach is not without its obstacles. Principally, there are ongoing concerns with the route of administration and viral vector specificity, both of which may result in potential systemic exposure and off-target effects of the vector. Besides off-target effects, there are also concerns of transduction efficiency, as well as, host or patient immunogenic responses. Because of these concerns, *ex vivo* gene therapy has become an alternative and secondary approach to *in vivo* gene therapy. *Ex vivo* therapy utilizes tissue or cells isolated from a patient or donor. These isolated cells are treated with vector containing the

therapeutic gene in culture, and then transplanted back into the patient or recipient. The most advancement has been made with cancer immunotherapies and the transduction of CAR-T cells. In the recent years, successful vector transduction of isolated respiratory cells from PCD and cystic fibrosis patients are encouraging [80,89,99], opening the door for future transplantation studies. In the context of olfactory ciliopathies, *ex vivo* gene therapy could serve as an alternative long-term solution. In addition to the potential hurdles of *in vivo* therapy, there is a biological limitation to the treatment. Mammalian OSNs are constantly turning over, where the lifespan of an individual OSN lasting anywhere from 60-90 days [106,107]. Therefore, there would be a decrease in efficacy of the *in vivo* treatment over time as OSNs turn over. A possible solution would be isolation and treatment of the OE stem cell population, and subsequent transplantation back into the host. Proof-of-principle experiments were successful in the isolation and transplantation of globose basal cells and horizontal basal cells (HBCs), the mitotically active and inactive multipotent progenitor stem cell populations of the OE, respectively [108–110]. Furthermore, these grafted cells demonstrated the capacity of generating multiple cell types, including OSNs, as well as the supporting sustentacular cells. This *ex vivo* strategy may also be useful for ciliopathies that might affect the olfactory stem cell populations. Recently, the HBC population were shown to have primary cilia [8]. While HBC-specific loss of ciliation did not affect the establishment and maintenance of the OE, loss of ciliation did inhibit the ability of the OE recovery after lesion. These observations suggest a possible ciliopathy phenotype outside of the OSN cilia, and susceptibility of ciliopathy patients to OE injury. Thus, *ex vivo* gene therapy may be a viable option for restoring proper ciliation and ciliation function within the HBCs. However, subsequent studies will need to be performed to examine the olfactory stem cell penetrance in patient relevant ciliopathy models.

Current Therapies and Future Challenges

Currently, there is no clinically approved universal treatment to directly address ciliopathies. Due to the complexity and pleiotropic nature of many ciliopathies, the majority of the existing treatments address the symptoms that would be considered most debilitating. These immediate treatments include surgery for polydactyly, weight management to address obesity, vision aids and mobility training for vision loss, special education for learning disabilities, and kidney transplants for renal dysfunctions. In regards to ciliopathy-induced olfactory dysfunctions, management of olfactory dysfunction is possible where there is residual olfactory function [18]. Under these circumstances, patients would follow a smell training regimen that would maximize use of the remaining sensory perceptions. Smell training has demonstrated improved detection threshold and olfactory sensitivity for patients with traumatic anosmia [111,112]. Similar improvements were observed in patients following post-infection induced olfactory loss [113,114]. In addition, patients undergoing the smell training have exhibited return of neuronal activity in olfactory areas within the brain suggesting a partial restoration of central function [115]. However, smell training remains a symptomatic management of olfactory dysfunction and does not directly address the cause nor prevent disease progression. This option is also not applicable to patients who are anosmic, or complete loss of olfaction. Therefore, a targeted and long-term therapeutic solution is still necessary in severe cases.

In many ways, the peripheral olfactory system is privileged as the tissue is readily accessible and amenable to direct treatment. Because of this combination of isolation and accessibility, potential small molecule and/or gene therapeutic treatments can bypass many of the complications associated with systemic delivery, tissue specificity, dosage, and invasiveness. However, the accessibility and efficiency of the treatment could be impeded by the presence of mucus within the nasal cavity. In studies of PCD, where there is excessive mucosal accumulation, lentiviral-mediated transduction in the nasal cavity was severely reduced [88]. A similar problem was encountered in cases of cystic fibrosis, where the mucus acted as barrier for AAV and adenoviral vectors [116–118]. In such cases, there is a need to physically disrupt the mucosal accumulation or develop mucosal penetrating particles to assist in small molecule or vector penetrance.

For the most part, ciliopathies follow standard Mendelian genetic inheritance. However, some studies suggest a more complicated inheritance scheme where the disease or symptoms result from accumulated mutations across different genes [119]. This idea of the polygenic causes of ciliopathies was observed in patient populations of BBS where mutations in both *Bbs6* and *Bbs2* genes contribute to the disease phenotypes [120]. Mutational load was observed in other BBS patients, where *Bbs4* mutations were accompanied with mutations in *Bbs1* and *Bbs2* [121]. With the involvement of multiple genetic mutations, the standard gene therapeutic approach of restoring a single gene may not be sufficient to restore ciliation or reverse the phenotypes. While co-infection of the same cell with different viral vectors is possible [9], the efficacy of a such a treatment is unknown. As such, alternative approaches may be required to address polygenic mutations.

At this time, the most viable treatment option for olfactory ciliopathies is through gene therapies. This is largely due to the compelling evidence of adenoviral and AAV-mediated delivery of wildtype genes to restore OSN ciliation in ORPK and BBS1 knockout mice [21,22]. However, there are still questions regarding whether this treatment is translatable to other ciliopathy models and to patients. The primary concern is the immunogenicity and toxicity of the treatment, which seem well tolerated in mouse models [22]. This is partially mitigated by the limited immunogenic response observed in cystic fibrosis studies where vector-related symptoms did not manifest in low doses of aerosolized adenoviral vectors [122]. Together, these observations suggest that the nasal cavity is one of the few areas that are immune-privileged. Another potential hurdle of gene therapy is the presence of a pre-existing immunity to adenoviral and AAV vectors. Pre-existing immunities diminishes the infection of efficiency of the several vectors, resulting in an overall reduction in the efficacy of the treatment [123]. One practical solution to this problem would be to simply change to another serotype, but the occurrence of pre-existing immunity within the olfactory system has yet to be documented.

CONCLUSION

To date, the field has made significant progress understanding the structure and function of cilia in the olfactory system while work examining the penetrance of ciliopathies in this tissue continues. The potential biological treatment options are compelling, while additional studies are required to examine the direct effect of small molecules on olfactory cilia.

However, this does not detract from the most intriguing and promising finding that is the ability to restore ciliation and cilia function following gene therapy. Future work is needed to optimize and streamline the delivery, improve the infection efficiency, and increase the cell specificity. With the successes observed thus far, treating ciliopathy-induced olfactory dysfunction may provide novel insights into developing improved treatments for other ciliopathy-afflicted organ systems, as well as other congenital causes of olfactory dysfunction.

Supplementary Material

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LIST OF ABBREVIATIONS

OSN	Olfactory Sensory Neuron
OE	Olfactory Epithelium
GPCR	G-Protein Coupled Receptor
HBC	Horizontal Basal Cell
IFT	Intraflagellar Transport
BBS	Bardet Beidl Syndrome
LCA	Leber Congenital Amaurosis
PCD	Primary Cilia Dyskinesia
PKD	Polycystic Kidney Disease
ADPKD	Autosomal Dominant Polycystic Kidney Disease
ORPK	Oak Ridge Polycystic Kidney
AAV	Adeno-associated Virus
ZFN	Zinc-finger Nuclease
TALEN	Transcription Activator-like Effector Nuclease
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats

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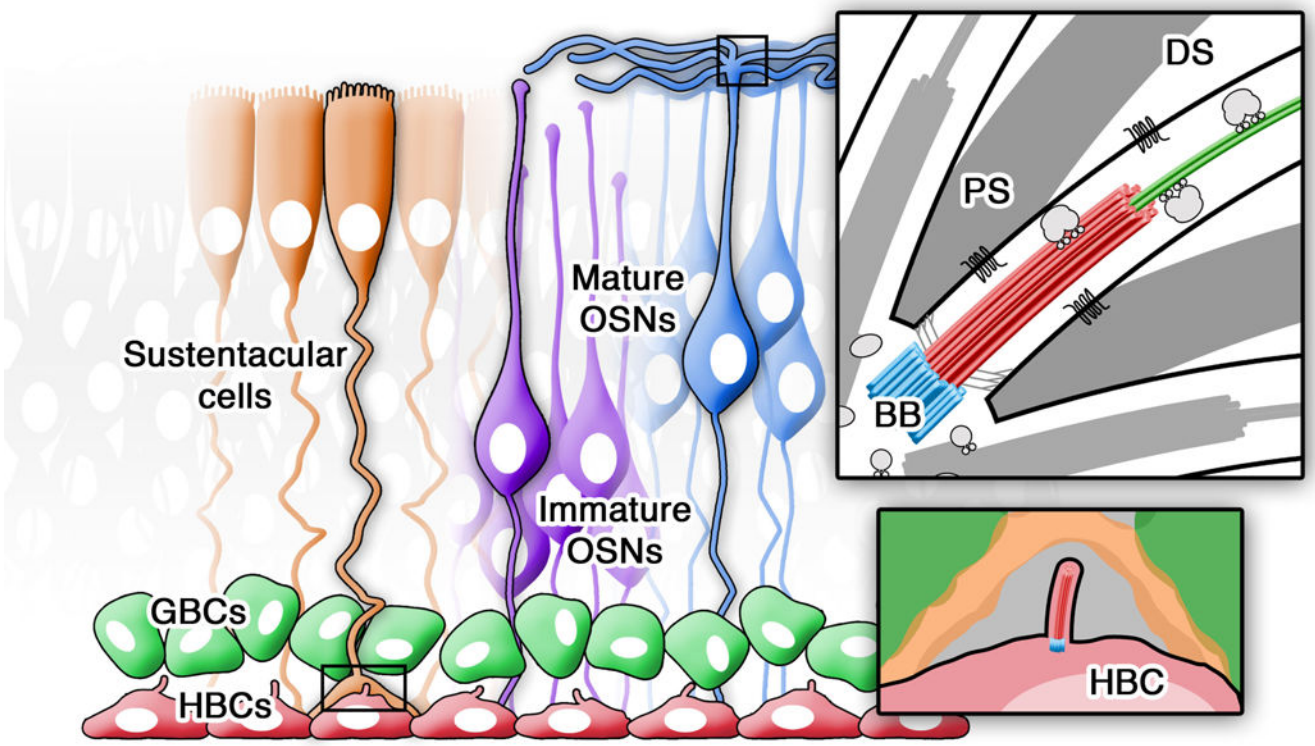

Figure 1. Olfactory epithelium cell types

Diagram depicting the major cell types present within the mammalian olfactory epithelium. At the basal surface are the ciliated horizontal basal cells (HBCs; red) and globose basal cells (GBCs; green). At the apical surface are the sustentacular cells (orange), and immature (purple) and multiciliated mature (blue) olfactory sensory neurons (OSNs). (Inset-top) Structural organization of OSN cilia depicting basal bodies (BB), proximal segment (PS), distal segment (DS), including trafficking of ciliary proteins and membrane bound G-protein coupled odor receptors. (Inset-bottom) Magnification of the HBC primary cilia, which has a role in OE neurogenesis.

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Table 1

List of congenital olfactory dysfunctions

Known ciliopathies demonstrating olfactory dysfunction are listed #'s 1 through 10.

#	Disease	Known Genetic Mutations	Confirmed Olfactory Dysfunction	Known Cause of Olfactory Dysfuntion	References
1	Bardet-Beidl Syndrome (BBS)	BBS1, BBS1(M390R), BBS2, BBS3/ARL6, BBS4, BBS5, BBS6/MKKS, BBS7, TTC8, BBS9, BBS10, TRIM32, BBS12, CEP290/NPHP6	BBS1, BBS1(M390R), BBS2, BBS4, BBS6/MKKS, BBS8	Olfactory epithelium (cilia loss)	18, 20, 25, 124, 125, 126
2	Meckel-Gruber Syndrome (MKS)	MKS1, MKS2, MKS3/TMEM67, CEP290/NPHP6, BBS1, BBS4, NPHP3	MKS1, MKS3/TMEM67, CEP290/NPHP6	Olfactory epithelium (signal transduction)	19, 126, 127, 128
3	Joubert Syndrome	CEP290/NPHP6, ARL13b, INPP5e	CEP290/NPHP6	???	19, 126, 129, 130, 131
4	McKusick-Kaufman Syndrome	BBS6/MKKS	BBS6/MKKS	???	24
5	Refsum Disease	PEX7, PHYH	???	???	132, 133
6	Polycystic Kidney Disease (PKD)	PKD1, PKD2, NPHP3, IFT88	PKD1, PKD2, IFT88	Olfactory epithelium (cilia loss; IFT88)	21, 128
7	Primary Ciliary Dyskinesia (PCD)	DNAI1, DNAH5	???	Olfactory epithelium (airway obstruction)	134
8	Leber Congenital Amaurosis (LCA)	CEP290/NPHP6, GUCY2D, RPE65, SPATA7, AIPL1, LCA5, RPGRIP1, CRX, CRB1, NMNAT1, IMPHD1, RD3, RDH12, LRAT, TULP1, KCNJ13, GDF6, PRPH2, LCA9, LCA3	CEP290/NPHP6	Olfactory epithelium (signal transduction; airway obstruction)	19, 126, 135
9	Jeune Syndrome	IFT80, DYNC2H1, WDY19, IFT40, TTC21B	???	???	136
10	Nephronophthisis	CEP290/NPHP6	CEP290/NPHP6	???	126
11	Kallmann's Syndrome	KAL1, AKAP2, FGF8, FGFR1, KISS1R, PROK2/ PROKR2, NELF, TAC3, TACR3, WDR11	KAL1, FGFR1, PROK2/ PROKR2, FGF8, CHD7, WDR11, NELF	Olfactory bulb development	137, 138, 139, 140, 141, 142
12	CHARGE Syndrome	CHD7	CHD7	???	143, 144
13	Usher Syndrome	USH1C, USH1B/MYO7A, CDH23, USH2/USH2A, PCDH15, CLRN1, SANA, USH1H, VLGF1b, DFNB31, PDZD7, USH3A	USH1	Olfactory bulb development	145, 146, 147, 148, 149
14	Microcephalic osteodysplastic primordial dwarfism type II (MOPDII)	PCNT	???	Olfactory bulb development	150
15	Seckel Syndrome	ATR, PCNT, CENPJ	???	???	21, 150, 151
16	Bosma Arhinia Microphthalmia	PAX6	???	Olfactory epithelium (airway obstruction); Olfactory bulb development	152, 153
17	Endocrine-Cerebro-Osteodysplasia	ICK	???	Olfactory bulb development	154

#	Disease	Known Genetic Mutations	Confirmed Olfactory Dysfunction	Known Cause of Olfactory Dysfuntion	References
18	Mohr-Majewski Syndrome	TCTN3	???	Olfactory bulb development	155, 156, 157
19	Isolated Congenital Anosmia	CNGA2, TENM1	CNGA2, TENM1	Olfactory epithelium (signal transduction)	158, 159, 160
20	Congenital Indifference to Pain	SCN9A	SCN9A	Olfactory epithelium (action potential propagation)	161