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Pathogenesis and Treatment of Usher Syndrome Type IIA

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Abstract: Usher syndrome (USH) is the most common form of deaf-blindness, with an estimated prevalence of 4.4 to 16.6 per 100,000 people worldwide. The most common form of USH is type IIA (USH2A), which is caused by homozygous or compound heterozygous mutations in the *USH2A* gene and accounts for around half of all USH cases. USH2A patients show moderate to severe hearing loss from birth, with diagnosis of retinitis pigmentosa in the second decade of life and variable vestibular involvement. Although hearing aids or cochlear implants can provide some mitigation of hearing deficits, there are currently no treatments aimed at preventing or restoring vision loss in USH2A patients. In this review, we first provide an overview of the molecular biology of the *USH2A* gene and its protein isoforms, which include a transmembrane protein (TM usherin) and an extracellular protein (EC usherin). The role of these proteins in the inner ear and retina and their impact on the pathogenesis of USH2A is discussed. We review animal cell-derived and patient cell-derived models currently used in USH2A research and conclude with an overview of potential treatment strategies currently in preclinical development and clinical trials.

Key Words: Usher syndrome, USH2A, usherin, gene therapy, antisense oligonucleotide

(*Asia Pac J Ophthalmol (Phila)* 2022;11:369–379)

USHER SYNDROME

Usher syndrome (USH) is the most common form of deaf-blindness, with an estimated worldwide prevalence of 4.4 to 16.6 per 100,000 people.[1,2] Usher syndrome is classified into 4 types, designated USH1, USH2, USH3, and USH4 that are further categorized into at least 11 subtypes associated with autosomal recessive mutations in specific genes. All types of USH lead to sensorineural hearing loss and visual dysfunction, and can be clinically distinguished according to the severity and onset of hearing loss, vestibular dysfunction, and vision deficits. USH2 is the most common form, accounting for approximately 50% of USH cases.[3,4] USH2 patients show moderate to severe hearing loss from birth, with onset of retinitis pigmentosa (RP) in the second decade of life and variable vestibular involvement.[5] USH2 is caused by homozygous or compound heterozygous mutations in 1 of 3 genes (*USH2A*, *ADGRV1*, and *WHRN*), which encode components of the USH2 protein complex. Loss of any 1 of these 3 proteins leads to the USH subtypes USH2A, USH2C, and USH2D, respectively.[6] An USH2B locus on chromosome 3p23-24.2 was proposed in 1999,[7] however, the authors later withdrew this classification after further analysis.[8]

The *USH2A* gene encodes 2 known isoforms of the usherin protein; a large single-pass transmembrane (TM) protein and a smaller extracellular (EC) protein lacking the C-terminal TM region (Figs. 1A, B). Usherin is required for the development of cochlear hair cells of the inner ear and long-term maintenance of retinal photoreceptors.[9] In addition to USH2, mutations in *USH2A* can also lead to either nonsyndromic autosomal recessive RP or pericentral retinal degeneration (PRD) without hearing defects.[10–12] To date, no curative treatment has been established for USH, however, a number of promising approaches are being developed for clinical implementation, including antioxidant therapy,[13] splice-altering antisense oligonucleotides (AOs), gene replacement, and gene-editing therapies. This review will explore the molecular pathogenesis of Usher syndrome type IIA (USH2A, OMIM 276901) and outline potential treatment strategies currently in development.

USH2A GENE, TRANSCRIPTS, AND PROTEINS

The *USH2A* gene (OMIM 608400, NCBI Gene ID: 7399) was first identified in 1998 through characterizing mutations in a gene associated with USH2A.[14] Only the shorter *USH2A* isoform was reported at the time of first discovery, and the longer isoform that includes an additional 51 novel exons at the 3′ end was identified in 2004.[15] The human *USH2A* gene is located within an 800 kb region of chromosome 1q41, consisting of a total of 72 exons with introns ranging from 0.1 to 78 kb. The 2 protein-coding transcript variants of *USH2A* identified in humans are shown in Figure 1 and Table 1. The shorter transcript includes 21 exons and encodes usherin protein isoform A, consisting of 1546 amino acids and is 170 kDa in size, while the longer 72 exon

Submitted March 24, 2022; accepted May 27, 2022.

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The authors have no conflicts of interest to declare.

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ISSN: 2162-0989
DOI: 10.1097/APO.0000000000000546

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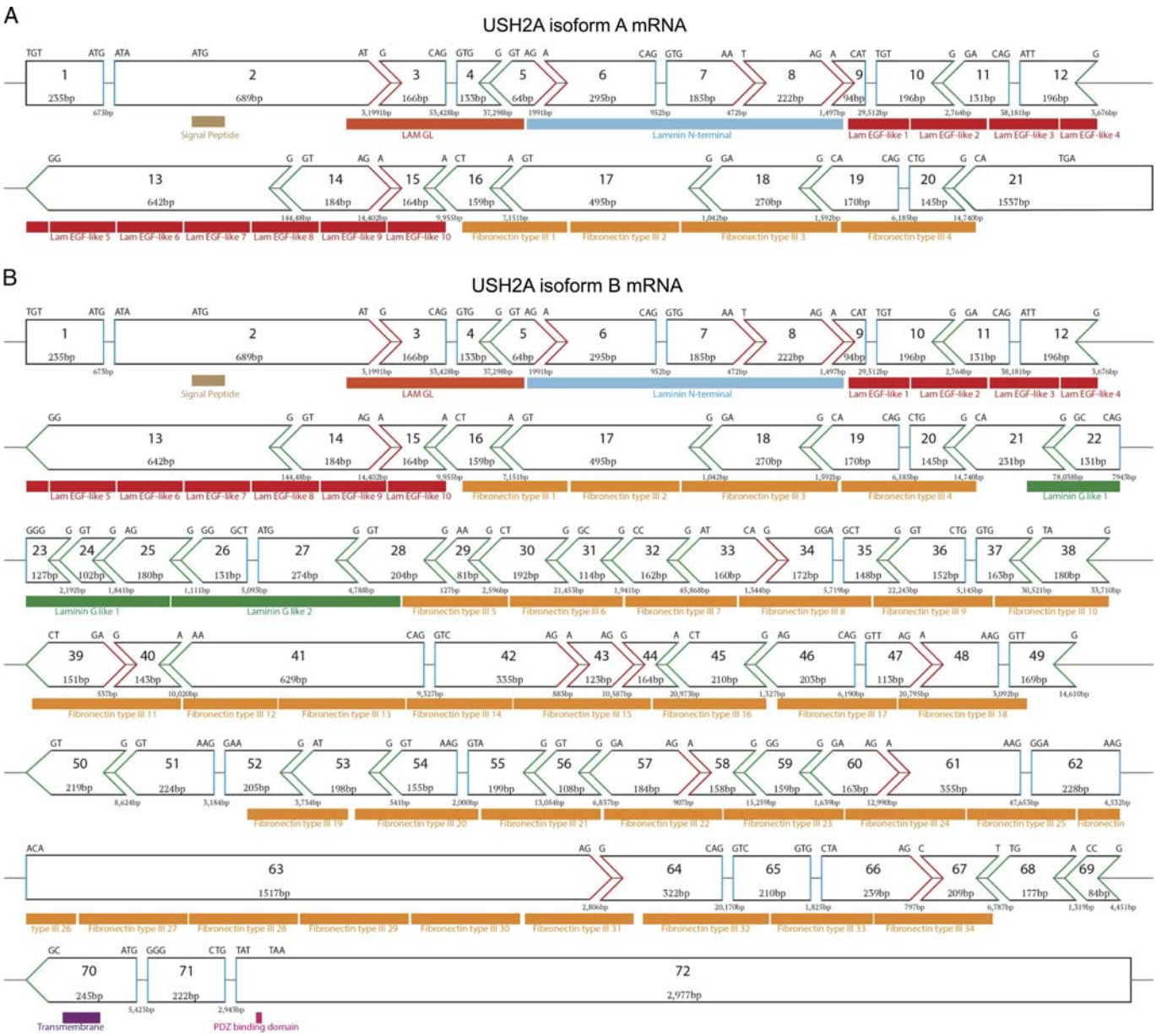

FIGURE 1. The coding exons and the relative position of protein domains of USH2A isoform A and B. mRNA indicates messenger ribonucleic acid.

transcript encodes usherin protein isoform B, with 5202 amino acids and a molecular weight of 580 kDa. These 2 variants are alternatively spliced at their 3′ ends. The longer transcript uses an alternative donor splice site within exon 21, resulting in a 14 base pair truncation at the 3′ end of exon 21 that removes the usherin isoform A stop codon.[15] In addition, an alternative transcript was reported to be expressed in the mouse inner ear, but not in the retina. The inner ear transcript includes an extra exon encoding 24 additional amino acids between exons 70 and 71 and sequence analysis predicted the utilization of this exon in human, rat, cow, dog, and zebrafish genomes.[16]

MUTATION SPECTRUM, GENOTYPE AND PHENOTYPE

USH2A variants include all types of mutations: insertions, deletions, nonsense, and missense mutations, large rearrangements, duplications, deep intronic mutations, and variants affecting splicing (USHbases; http://www.lovd.nl/USH2A).[10] The first missense mutations reported in the *USH2A* gene to be associated with recessive RP without hearing loss were identified in 2000.[11] Mutations in the *USH2A* gene as well as being the most common cause of Usher syndrome (50% of all cases)[3] are also one of the most

TABLE 1. Transcript Variants of USH2A and Encoded Proteins

Usherin Isoform	Transcript Variant	Exon Counts	Nucleotide Counts	Protein	Amino Acids	Size
EC usherin	NM_007123.6	21	6372 bp	NP_009054	1546	170 kDa
TM usherin	NM_206933.4	72	18,938 bp	NP_996816	5202	546 kDa

EC indicates extracellular; TM, transmembrane.

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common causes of nonsyndromic RP (19%–23% of autosomal recessive RP)[17] and PRD.[12] More than 600 different mutations in *USH2A* have been reported; analysis of the mutation spectrum of the entire long isoform revealed that pathogenic mutations are distributed throughout the entire *USH2A* gene locus, with around 60% occurring in the 51 exons specific to the long isoform.[18,19] A frameshifting microdeletion in exon 13, c.2299delG (p.Glu767fs), is the most common pathogenic variant. This variant, thought to be derived from an ancestral founder,[20] is present in 16% to 30% of USH2A patients.[18,21]

Some mutant alleles are found only in individuals with the nonsyndromic retinal disease. These retinal-specific pathogenic *USH2A* variants, found in patients with nonsyndromic RP but not in USH2 patients, located in the exons encoding usherin EC domains.[22] The presence of at least one retinal-specific *USH2A* mutant allele in a patient with *USH2A*-associated disease results in normal hearing, at least in childhood. Retinal-specific *USH2A* variants include one of the common mutations, c.2276G > T (p.Cys759Phe), as well as other variants such as c.2802T > G (p.Cys934Trp), c.10073G > A (p.Cys3358Tyr), c.11156G > A (p.Arg3719His), c.12295-3T > A, and c.12575G > A (p.Arg4192His).[22]

USHERIN PROTEIN STRUCTURE, DOMAIN FUNCTIONS, AND BINDING PARTNERS

Usherin isoform B (TM usherin) is a large membrane bound protein consisting of multiple domains. The N-terminal EC domain is made up of repeating fibronectin type III (FN3) domains and laminin-like domains, including a laminin G-like jellyroll fold domain (LamGL), a laminin N-terminal domain (LamNT), laminin-epidermal growth factor-like domains (LE) and 2 laminin G domains (LamG). The C-terminus of TM usherin consists of a TM domain, followed by an intracellular domain with a PDZ binding motif (PBM). In contrast, the smaller usherin isoform A (EC usherin) consists of a portion of the EC domain containing LamGL, LanNT and LE domains and the N-terminal FN3 domains of TM usherin (Fig. 1).

LamNT domains function as calcium-dependent domains through which the laminin heterotrimers assemble to anchor to the cell membrane via the LamG domain. The LE domains form a rigid, rod-shaped structure stabilized by 4 disulfide bridges that prevents domain extension under mechanical stress.[23–25] The major portion of usherin is occupied by 34 repeats of FN3 domains that form 2 antiparallel β-sheets without disulfide bonds, that act as a shock absorbers by unfolding under mechanical tension and folding again when stress is released.[26] The single cytoplasmic PBM near the carboxy tail is the only known functional domain inside the cytoplasm, allowing usherin to interact with various PDZ domain-containing proteins involved in anchoring TM proteins to the underlying cytoskeleton.[27] Similar to the majority of single-pass TM proteins, TM usherin undergoes *N*-glycosylation that take places at asparagine amino acids throughout its EC region.[28] Recently, threonine 4999 of TM usherin was described as a phosphorylation site in K562 cells, a human erythromyeloblastoic leukemia cell line.[29]

The first identified binding partner of usherin was the basement membrane protein collagen IV, which was found to interact with EC usherin.[30] EC usherin is a component of Bruch membrane in the retina[31] and the basement membrane of the cochlea.[30] Mutations in one of the genes encoding the heterotrimers of collagen IV lead to Alport syndrome, the symptoms of which include hearing loss and eye abnormalities. EC usherin binds to the 7S domain of collagen IV through its LE domains. Reduction of collagen IV in the Alport mouse testis is associated with a similar reduction of EC usherin, suggesting that the usherin/collagen IV interaction is important in stable integration of usherin in the basement membrane. Another basement membrane protein shown to interact with usherin LE domains is fibronectin. Both collagen IV and fibronectin were found to colocalize with EC usherin in Bruch membrane,[32] however, it remains unclear whether the lack of EC usherin in cochlear and retinal basement membranes contributes to disease pathogenesis.

The class I PBM present at the C-terminal of the TM usherin cytodomain enables interaction with PDZ domain-containing proteins. Such proteins act as organizers of molecular complexes, and some are involved in anchoring TM proteins to the underlying cytoskeleton.[27] TM usherin forms a protein complex with 2 other proteins encoded by USH2-associated genes; *ADGRV1* (encoding adhesion G Protein-Coupled Receptor V1) and *WHRN* (encoding whirlin).[33] TM usherin and ADGRV1 do not interact directly, and both whirlin and the USH modifier protein PDZD7 are required for complex formation, where whirlin and PDZD7 bind to

TABLE 2. Usherin and Interacting Proteins

Interacting Protein	Involved Domain of the Interacting Protein	Involved Domain of Usherin	References
USH-related proteins			
Myosin VIIA	My TH4-FERM	Cytoplasmic region	37
Harmonin	PDZ1	PBM	36
Whirlin	PDZ1 and PDZ2	PBM	16,33,35
PDZD7	PDZ1 and PDZ2	PBM	38
ADGRV1	indirect interaction via whirlin and PDZD7		33
Non–USH-related proteins			
Collagen IV	7S domain	Loop b of LE domain	30
Fibronectin	Not known	Loop d of LE domain	32
Vezatin	Not known	Cytoplasmic region	37
NLP isoform B	IF domain	Cytoplasmic region	39

LE indicates laminin EGF-like domains; My TH4-FERM, myosin tail homology 4-protein 4.1, ezrin, radixin, moesin domain; NLP, ninein-like protein; PBM, PDZ binding motif; USH, Usher syndrome.

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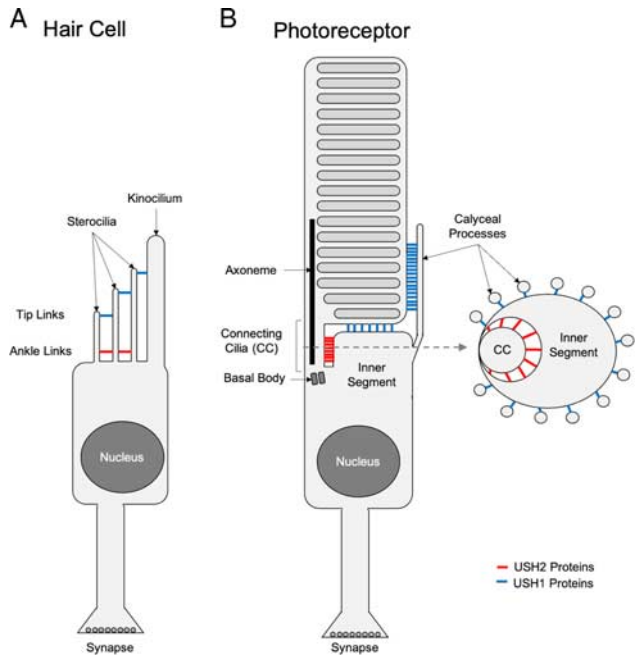

FIGURE 2. Hair cell (A) and photoreceptor (B) structure with location of USH2 (red) and USH1 (blue) protein complexes indicated.

usherin and ADGRV1, respectively. The direct interaction of usherin and whirlin was first shown in vitro[34] and the biological relevance of this interaction was confirmed by the absence of whirlin at the photoreceptor periciliary membrane of *Ush2a* knock-out mice.[35] TM usherin and ADGRV1 were also shown to form complexes with the USH1 protein harmonin, encoded by the *USH1C* gene[36] (Table 2).

USHERIN IN THE INNER EAR

The human ear is divided into 3 main parts: the outer ear, the middle ear, and the inner ear. The inner ear contains the cochlea and vestibular labyrinth for sensing sounds and balance, respectively. The organ of Corti, the sensory epithelium of hearing within the cochlea, has 3 rows of outer hair cells and 1 row of inner hair cells. The vestibular system consists of 2 types of sensory hair cells (type I and type II). Hair cells in both cochlear and vestibular system are sensory receptors possessing a specialized mechanosensitive structure, the hair bundle, on the apical surfaces of the cells. The hairs of the hair bundle, erroneously termed stereocilia,[40] are rigid actin-based microvilli-like structures arranged in a staircase array with rows of increasing height toward the centrally located microtubule-based genuine cilium, the kinocilium. The kinocilium provides structural polarity on the hair bundle during morphogenesis and disappears in mature hair cells. The shorter stereocilia in each row are connected with the adjacent stereocilia in the taller row through the tip links and ankle links at apex and base, respectively.[41] Defects in the morphogenesis, organization, and stability of stereocilia in the hair bundles result in impaired mechanotransduction causing sensorineural hearing loss and balance defects.

In the cochlea, usherin is expressed in the developing hair cells and in the spiral ganglion cells.[9,34,37] In developing hair cells, USH2 complexes form ankle links at the base, while USH1 proteins form tip links at the distal ends of adjacent stereocilia (Fig. 2A). TM usherin was detected in stereocilia ankle links in the inner ear hair cells of embryonic day 20 mice, and while it started to disappear by postnatal day 10 in the auditory hair cells, expression of TM usherin persisted in the hair cells of the vestibular system.[16] This transient expression pattern of TM usherin expression in auditory hair cells is correlated with the maturation of stereocilia and the disappearance of the kinocilia. The transient expression of TM usherin protein in stereocilia ankle links suggests an important role for usherin in maintaining cochlear hair cell ultrastructure during development. Hearing loss in USH2 patients is present from birth and affects the perception of higher frequencies.[6,42–44] Histopathological studies of USH inner ears have demonstrated degenerative changes in the organ of Corti, hair cell loss, as well as atrophy of the stria vascularis and spiral ganglion neurons.[45] Vestibular dysfunction may also be present in up to one-third of USH2 patients.[5,46]

USHERIN IN THE RETINA

The retina is a multilayered neural tissue lining the inner posterior surface of the eye. The outer layers of the retina consist of Bruch membrane and the retinal pigment epithelium (RPE), which lie adjacent to the light-sensitive outer segments of retinal cone and rod photoreceptors. Photoreceptors are composed of an outer and an inner segment joined by a thin connecting cilium, a cell body containing the nucleus and a synaptic terminal connecting to the bipolar neurons of the inner retina. The outer segments of both rod and cones are filled with stacks of flattened, membranous discs that contain components of the phototransduction pathway. The connecting cilium linking the outer and inner segments is important for the intracellular trafficking of molecules and proteins between the 2 segments, and is an important site of subcellular localization for TM usherin and other USH proteins[47] (Fig. 2B).

TM usherin is the predominant form expressed in photoreceptor cells, where it localizes to a distinct region in the apical inner segment that wraps around the connecting cilium, forming a semiencircled recess.[9] USH2 complexes containing TM usherin, ADGRV1, and whirlin localize in the periciliary membrane complex (PMC) in photoreceptors.[35,48] Deletion of the PBM in the cytoplasmic tails of usherin weakens this interaction and impairs the localization of other USH protein complex partners, *adgrv1* (USH2C) and *whirlin* (USH2D) at the photoreceptor periciliary region of *ush2a* mutant zebrafish models.[49] EC usherin is localized in Bruch membrane,[31] however it remains unclear whether this isoform plays a role in USH2 pathogenesis.

USH2A-associated retinopathy is typically diagnosed in the patient's teens, presenting as an RP phenotype, with loss of rods leading to early symptoms of nyctalopia followed by loss of cone photoreceptors and gradual constriction of the visual field.[50,51] Some cases of *USH2A*-associated retinopathy may present as late as the fifth decade with PRD phenotype and pericentral ring scotoma with minimal progression or night vision impairment. The progressive degenerative phenotype suggests USH2 complexes play a crucial role in maintaining photoreceptor homeostasis, similar to other RP genes that express proteins involved in connecting cilia function. It remains to be determined if modifier genes may limit the severity of *USH2A*-associated retinopathy to a pericentral distribution.

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TABLE 3. USH2A Animal Models

Animal Model	Genotype	Phenotype	References
Ush2a$^{-/-}$ mouse	Targeted disruption of exon 5 of *Ush2a* by a standard gene-targeting technique	Retinal degeneration at 20 months of age, hearing impairment tested at 4 months of age with nonprogressive moderate hearing loss, no signs of vestibular dysfunction	9
ush2a$^{-/-}$ knockdown zebrafish	Exon 6 skipping by targeting the splice donor site of exon 6 by morpholino AO	Photoreceptor degeneration	38
ush2a^{rmc1} zebrafish	c.2337_2342delinsAC (p. Cys780GlnfsTer32) by CRISPR/Cas	Early onset of photoreceptor dysfunction Increased photoreceptor apoptosis Impaired localization of other USH protein complex members (Adgrv1 and whirlin) at the photoreceptor periciliary region	49
ush2a^{b1245} zebrafish	c.15520_15523delinsTG (p.Ala5174fsTer) by CRISPR/Cas9		
ush2aPE40/*PE40* zebrafish (humanized knockin model)	Deep-intronic c.7595-2144A > G mutation in intron 40 by CRISPR/Cas9	Poor recognition of splice site for human *USH2A* pseudoexon 40 (7.4% ± 3.9%) splice site correction by pseudo exon 40-targeting AO	52

AO indicates antisense oligonucleotides.

ANIMAL MODELS OF USH2A

A number of different animal models for Usher Syndrome, both engineered and naturally arising, have been characterized (Table 3). Although USH1 and USH2 mouse models both show hearing deficits, only USH2 models have been reported to manifest any retinal degeneration.[9,35] The lack of retinal phenotype in USH1 mouse models has been attributed to the lack of calyceal processes in mouse photoreceptors, which are an important site of USH1 protein localization in humans, macaques, fish, and frogs.[48] Unlike the USH1 mouse models that usually have normal vision, mouse models lacking usherin or whirlin show both hearing deficits and late-onset retinal degeneration.[9,35,53–55]

The first *Ush2a* knockout mouse model was generated by microinjection of embryonic stem cells and targeted disruption of exon 5 of *Ush2a* into blastocysts.[9] These mice have normal morphology and numbers of photoreceptors at 10 months of age, but by 20 months over half the photoreceptor cells were lost and outer segments became disorganized. *Ush2a* knockout mice showed impaired hearing at higher frequencies, consistent with an observed loss of outer hair cells in the basal turn of the cochlea. Interestingly, loss of basal turn hair cells was previously reported in a human USH3 patient.[56] Inner ear hair cells of *Ush2a* knockout mice appeared normal, and no vestibular dysfunction was observed. The nonprogressive moderate hearing loss and progressive retinal degeneration observed in these mice is consistent with the disease presentations generally found in USH2 patients. The relatively late onset of visual dysfunction in these mice remains a moderate obstacle for preclinical studies by extending experimental time courses necessary for screening. However, histopathological changes, such as glial fibrillary acidic protein upregulation, are observable in the retina at much earlier timepoints and could potentially be used as markers of disease in the absence of overt photoreceptor degeneration.[9]

The Kunming mouse, KM$^{ush/ush}$, was proposed as a naturally occurring USH mouse model exhibiting moderate nonprogressive congenital deafness and early rapid retinal degeneration. Initially, the authors reported 25 mutations across 13 different exons in the *Ush2a* gene of KM$^{ush/ush}$ mice as unpublished data,[57] however later studies showed independent segregation of the hearing and vision loss phenotypes when mice were crossed with other strains. The visual phenotype of KM$^{ush/ush}$ mice was associated with the known rd1 mutation in the *Pde6b* gene, while whole exome sequencing uncovered a single-base pair deletion variant in the *Adgvr1* gene.[58] The CBA-2$^{ush/ush}$ mouse strain that lacks the rd1 variant does not show a retinal phenotype, but may provide a model for USH2C-associated hearing loss.

The first zebrafish model of *ush2a* knockdown was achieved by injection of a morpholino AO targeting the splice donor site of exon 6 in a study of the interaction between *pdzd7a* and *ush2a*.[38] A moderate degree of photoreceptor degeneration was observed in *ush2a* knockdown zebrafish, and became more prominent when treated with the combination of a higher dose of *ush2a* knockdown AO and a half-dose of a *pdzd7a* knockdown AO. Later, a humanized knockin zebrafish model was engineered to mimic the cryptic splicing of a pseudoexon (PE40) into human *USH2A* mRNA transcripts carrying the c.7595-2144A > G variant.[52] However, the PE40 splice sites were poorly recognized by the zebrafish splicing machinery resulting in ~7.4% ± 3.9% of total *ush2a* transcripts with the human PE40 sequence.

In 2018, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas-9) technology was used to generate 2 *ush2a* mutant zebrafish models, *ush2a*^{rmc1} and *ush2a*^{b1245}, with 2-base pair deletions introduced into exon 13 or 71, respectively. Truncated usherin was expressed in *ush2a*^{b1245} larvae and localized correctly to the periciliary region of photoreceptors, but usherin protein was not detectable in *ush2a*^{rmc1}. Larvae of both mutants showed exacerbated photoreceptor degeneration with constant light rearing and early-onset retinal dysfunction with reduced electroretinogram wave responses.[49] The *ush2a*^{rmc1} zebrafish model has recently proven useful in the preclinical evaluation treatments for USH2A patients.[59]

USH2A PATIENT-DERIVED CELL MODELS

A variety of patient-derived cellular models have been utilized in in vitro studies of USH2A. Vaché et al[60] reported nasal ciliated epithelial cells as a reliable cell model for anal-

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TABLE 4. Induced Pluripotent Cell Lines Generated From Patients Carrying USH2A Mutations

USH2A Mutations	Cells of Origin	Differentiation	References
c.12575G > A (homozygous)	Keratinocytes	Retinal precursor cells	63
c.8559-2A > G, c.9127_9129delTCC	Urine-derived cells	Retinal pigment epithelial cells and retinal organoids	64,65
c.2299delG, c.1256G > T	Peripheral blood mononuclear cells		66
c.2802 T > G, c.12560G > A, c.8559-2A > G	Peripheral blood mononuclear cells		67
c.8559-2A > G (homozygous)	Peripheral blood mononuclear cells		68
c.949C > A, c.1256G > T	Fibroblasts		69,70
c.2299delG, c.2276G > T	Fibroblasts		71
c.2299delG (homozygous)	Fibroblasts		72
c.2276G > T, c.7352C > T	Fibroblasts		73
c.2209C > T, c.8693A > C	Fibroblasts		74
c.2276G > T (homozygous)	Fibroblasts		75
c.2299delG (homozygous)	Fibroblasts	Photoreceptor progenitor cells	59

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ysis of Usher transcripts. Together with the other 2 USH2 genes (*ADGRV1* and *WHRN*), both isoforms of *USH2A* transcripts were detectable in nasal epithelial cells. In addition, the splicing defects associated with 4 *USH2A* variants were identified, which included: an acceptor site mutation (c.2168-1G > C) leading to deletion of 7 nucleotides at the 5′ end of exon 13 in the mature transcript; an acceptor site mutation (c.7595-8C > G) leading to inclusion of the last 7 nucleotides of intron 40 in the mature transcript; a 4-base pair duplication (c.4576_4579dupGGGT) leading to the deletion of 50 nucleotides at the 3′ end of exon 21; and a synonymous mutation (c.949C > A) creating a cryptic donor splice site within exon 6 leading to the exclusion of 193 nucleotides from the 3′ end of exon 6. The same group described another splicing defect caused by a deep intronic mutation (c.7595-2144A > G) resulting in the inclusion of pseudouexon 40 in the mature transcript.[61] The same splicing defect was seen in patient fibroblasts, however the AO-mediated splice site correction was performed in an in vitro minigene splice assay due to the extremely low expression of *USH2A* in fibroblasts.[62]

With the limited availability of USH2A patient retinal and cochlear tissues, induced pluripotent stem cells (iPSCs) have become an important potential source of patient-derived retinal and cochlear cells for in vitro analysis (Table 4). USH2A patient-derived iPSC lines have been established from many accessible cell types, including urine-derived cells,[64] peripheral blood mononuclear cells,[68] fibroblasts,[74] or keratinocytes.[63] Our group has produced iPSC lines from 2 USH2A patients harboring the c.949C > A and c.1256G > T variants.[69,70] We found that *USH2A* was expressed in iPSC, and recapitulated the c.949C > A splicing defect that results in internal truncation of exon 6 (unpublished data).

Generation of RPE and retinal organoid from USH2A-patient iPSC has provided a unique opportunities for gaining insights into the disease mechanisms associated with specific *USH2A* mutations.[63,65] For example, Tucker et al[63] investigated the effects of 2 variants on *USH2A* expression in patient-derived retinal organoids, providing functional pathogenicity evidence for protein misfolding and splicing defects in human retinal cells. More recently, Guo et al[64,65] showed marked defects in retinal organoid development, with disorganization of retinal layers, reduced laminin deposition, increased apoptosis, and decreased expression of genes associated with cilia and dopaminergic synapses in retinal cells derived from a patient with USH2A. Methods for the differentiation of inner ear cells from iPSC have also been developed,[76–78] however the application of these techniques to patients with USH2 has not yet been reported. The ability to generate affected tissues from accessible patient cell sources provides the opportunity to test novel therapies on the retinal and inner ear cells of the patients for whom the therapies are intended, providing an ideal in vitro model for preclinical screening.

CURRENT TREATMENTS FOR USH2A

To date, no curative treatment has been established for USH2A patients. Patients with USH mainly rely on early diagnosis and counselling to adapt themselves to dual sensory loss. In USH1 and USH2, deafness precedes visual impairment by a decade or longer. The delayed onset of visual symptoms often leads to a misdiagnosis of isolated deafness in early years. Since sign language may become difficult after visual deterioration, comprehensive molecular diagnosis and early bilateral cochlear implantation for USH1 are crucial for the acquisition of oral language and communication. In nonsyndromic RP, most of the photoreceptors may have already been irreversibly damaged by the time of diagnosis. In contrast, if USH syndrome can be diagnosed in a young patient with congenital hearing loss, before the development of RP in later life, photoreceptors could potentially be preserved by early neuroprotective interventions.

ANTIOXIDANT THERAPY

In RP, photoreceptor cell death initially involves the rod cells, with cone cell death beginning only after the majority of rod cells have degenerated and progressing more gradually. It has been suggested that the initial loss of rod photoreceptors reduces the retinal consumption of oxygen, leading to excessive tissue oxygenation and increased oxidative stress in cone cells.[13] Both animal models of RP[79–81] and human RP patients[82,83] show evidence of increased oxidative stress in the retina, suggesting this may be an important mechanism of cone cell death

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in RP. Supported by preclinical studies demonstrating that administration of antioxidants could reduce oxidative stress markers and preserve cone cells in mouse models of RP,[80,81] a recent clinical study examined the use of the antioxidant *N*-acetyl cysteine in the treatment of 30 patients with RP, 7 of whom had mutations in *USH2A*.[13] RP patients receiving oral *N*-acetyl cysteine showed improvements in best-corrected visual acuity over the 6-month treatment period, as well as reductions in aqueous oxidative stress markers, suggesting recovery of slowly degenerating cone cells in the macula. Although further trials are necessary, these promising results provide a strong basis for the further investigation of antioxidant treatments as a means of slowing secondary cone degeneration in all forms of RP, including Usher syndrome.

GENE REPLACEMENT THERAPY

With the recent clinical success of adenovirus-associated viral (AAV) vectors for the delivery of a functional copy of the *RPE65* gene for the treatment of Leber congenital amaurosis,[84] replacement of mutant genes in the retina has become a promising therapeutic approach for many types of inherited retinal diseases. However, *USH2A* and many other USH genes have very large coding sequences that exceed the packaging capacities of AAV vectors (4.8 kb), which has hampered the application of this technology to USH treatments. Initial attempts to overcome this limitation resulted in a Phase 1/2 clinical trial by Sanofi for USH1B using a lentiviral vector containing a *MYO7A* cDNA (UshStat or SAR241869), which was initiated in 2012 but suspended in 2017 and terminated in 2019. The results of this trial remain unpublished, but its early termination, together with the notoriously low photoreceptor targeting capabilities of lentiviruses[85] are not encouraging. In contrast, AAV has several desirable aspects as a therapeutic gene transfer vector, including the absence of natural infections associated with disease and availability of methods for scalable production and analytical quality control. The final product is highly stable and produces a high level of gene transfer to target tissues.[86] Furthermore, the safety profile of AAV is also attractive due to excellent tolerability and minimal inflammatory response in preclinical and clinical trials. For nondividing or slowly dividing cell targets such as photoreceptors and hair cells, this expression is long-lived due to the episomal nature of the AAV vector, facilitating a single injection therapeutic approach and leading to a durable effect, possibly lasting for the life of the patient. In the ear, AAV-based gene therapies have been used to demonstrate rescue of gene and protein expression and auditory function in mouse models of USH1 and USH3. Delivery of AAV vectors to the mouse inner ear has improved hearing and hair cell disorganization in the UHS1G mouse,[87] restoration of Clarin-1 protein and rescue of low-frequency hearing in the USH3 mouse,[88] and recovery of Harmonin protein expression in the USH1C mouse.[89] Despite these promising results, none of these therapies are currently in clinical trials, most likely due to the very low prevalence of USH1G, USH1C, and USH3 patients.

Driven by this desirable clinical profile, efforts to adapt the AAV-system for delivery of larger genes have led to the development of multivector AAV delivery systems, in which fragments of the gene coding sequence are incorporated into 2 or more AAV vectors. Coexpression of these gene fragments in transduced cells leads to recombination of partial transcripts into mRNAs containing the full gene coding sequence.[90–94] Although dual vector AAV therapy was initially reported to be inferior to lentiviral delivery for the *MYO7A* gene in the USH1B mouse retina,[93] studies in pigs indicate dual and triple vector AAV delivery can achieve retinal transduction efficiencies of 30%–50% of that achieved with a single vector.[91,94] Although there are no current USH2A clinical trials utilizing multivector AAV gene delivery, this may be a feasible future strategy for permanent restoration of usherin expression in the ear and eye.

AO THERAPY

Another promising approach to restoring usherin expression in USH2A patients is the targeted modulation of gene expression through splice switching AOs. AO are short synthetic nucleotide sequences designed to anneal to specific DNA or RNA targets. Using a variety of chemical modifications, different types of AOs have been developed for modifying gene expression through a range of different mechanisms. For example, splice-switching AOs synthesized using 2′-*O*-methyl bases on a phosphorothioate backbone or phosphorodiamidate morpholino chemistries can be targeted to splicing motifs, where they function as steric blocking agents that prevent the binding of splicing factors and alter splice site selection.[95] A number of splice-switching AO drugs, including Eteplirsen, Golodirsen, and Casimersen have been granted the US Food and Drug Administration accelerated approval for clinical use in Duchenne muscular dystrophy patients. These drugs induce the skipping of out-of-frame dystrophin exons that flank pathogenic frame shifting deletions to restore the reading frame during pre-mRNA splicing. The resulting dystrophin proteins, while internally truncated, is correctly localized in patient muscle and retains partial function, thereby providing some disease amelioration.[96,97] In addition, the exceptional development of Milasen, an AO designed and approved to correct a rare splicing defect in the *CLN7* gene in <12 months, demonstrates how these drugs can be tailored to address disease caused by individual variants.[98]

The first AO-mediated molecular therapy for the *USH2A* gene was proposed in 2016. A deep-intronic mutation (c.7595-2144A > G) creates a cryptic splice donor site in intron 40 resulting in the incorporation of a 152-bp pseudoexon (PE40) into the mature transcript. Inclusion of PE40 causes a frameshift in the coding sequence that introduces to a premature termination codon in exon 41 (p.Lys2532Thrfs*56).[61] AOs composed of 2′-*O*-methyl modified bases on a phosphorothioate backbone were designed to target the PE40 splice acceptor site and/or exonic splice enhancer regions induced significant splice correction in both patient-derived fibroblasts and in a minigene splice assay.[62] Other splice-altering mutations in *USH2A*, such as the c.949C > A variant,[60] may be amenable to AO-mediated interventions, however the rarity of specific pathogenic mutations remains an impediment to the clinical development and implementation of these personalized medicines.

More recently, AO-mediated exon skipping strategies, similar to those used in Duchenne muscular dystrophy patients, have been proposed as potential therapeutic strategies for targeting *USH2A* mutations. The long *USH2A* transcript

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includes 25 exons (Fig. 1) that can be skipped without disrupting the *USH2A* reading frame. Of these potentially skippable exons, exon 13 has recently been reported as a viable therapeutic target for this strategy.[59] Two of the most common *USH2A* variants, c.2299delG and c.2276G > T, are located within exon 13, and together account for an estimated 35% of pathogenic *USH2A* alleles.[10] In 2021, Dulla and colleagues reported the preclinical validation of QR-421a, an AO designed to induced skipping of exon 13 in *USH2A* transcripts. This 21 nucleotide RNA molecule, synthesized with a 2′-*O*-(2-methoxyethyl) ribose sugar modification on a phosphorothioate backbone, was shown to induce *USH2A* exon 13 skipping in photoreceptor progenitor cells derived from patient-iPSC harboring homozygous c.2299delG variants. Additionally, analogues of QR-421a were designed to induce exon 13 skipping in the *USH2A* homologues in zebrafish (*ush2a*) and mice (*Ush2a*). In the *ush2a*^{rmc1} zebrafish, which lacks usherin protein due to a frameshifting deletion mutation in exon 13, AO treatment induced exon 13 skipping, restoring the *ush2a* reading frame and resulting in the production of an internally truncated usherin protein. Importantly, AO treatment restored electroretinogram responses in *ush2a*^{rmc1} zebrafish to wild-type levels, demonstrating the truncated protein retained sufficient function to achieve a therapeutic benefit. The mQR-421a mouse surrogate AO was shown to induce skipping of exon 12 in wild-type mice (homologous to exon 13 in humans and zebrafish) for up to 6 months after intravitreal injection and gymnotic retinal uptake. QR-421a is currently undergoing clinical trials (ClinicalTrials.gov: NTC03780257) as the first AO therapy for *USH2A* mutations.

GENE-EDITING TECHNOLOGIES

Another potential therapy being explored is gene correction by CRISPR/Cas-9 targeted genome repair. In 2017, the most prevalent mutation was corrected using CRISPR/Cas9 gene editing in fibroblasts from an USH patient carrying homozygous c.2299delG mutations in *USH2A*.[99] Successful in vitro mutation repair was demonstrated using locus-specific RNA-Cas9 ribonucleoproteins with subsequent homologous recombination repair induced by an engineered template. Later, the mutation was similarly repaired in iPSC derived from several patients harboring the same variant.[66,100] The gene editing in the patient-derived iPSC corrected the accumulation of mRNA associated with the c.2299delG mutation.[100]

Although CRISPR/Cas9 facilitated the high efficiency introduction of double-stranded DNA breaks (DSBs) into specific targeted sequences, early CRISPR-Cas9 gene-editing techniques relied on low-efficiency homology directed repair (HDR) mechanisms for integration of the repair template into the targeted DSB. While this made for a reliable method for introducing gene sequence modifications in vitro, the translational potential of CRISPR-Cas9 gene repair was limited by its propensity to induce indel mutations in the very genes it is supposed to repair.[101] Further development of CRISPR-Cas systems led to improvements, such as dual nickase approaches that introduce single strand nicks instead of DSBs, reducing the frequency of off-target effects and expanded capabilities, such as RNA targeting (eg, Cas13)[102] and transcriptional modulation (eg, dCas9-VPR, dCas9-KRAB).[103,104] Recently, the development of base and prime editing systems renewed hopes for a clinically translatable gene correction technique. Instead of relying of HDR of a cotransfected repair template, second generation CRISPR base editors (BEs) and prime editors (PEs) contain their own machinery for introducing genetic modifications directly.[105] In the case of BE, Cas proteins fused with DNA base conversion enzymes can mediate all possible base changes, making BEs a highly attractive candidate for repairing single nucleotide mutations. However, since base conversion can occur on all amenable nucleotides within the 6-base pair to 7-base pair editing window of the BE, the sequence context of individual variants must be carefully assessed to determine the suitability of this approach. On the other hand, PEs utilize a Cas protein fused to a reverse transcriptase and guide RNAs fused to a repair template containing the desired genetic modification. Upon binding to the targeted sequence, PEs introduce a nick into the DNA, then repair the targeted site by reverse transcription from the repair template. Since a single PE enzyme can mediate the introduction of all single nucleotide conversions as well as small insertions and deletions, PE may provide a more flexible solution for clinical gene editing applications.

Several clinical trials utilizing CRISPR gene-editing technologies are currently underway,[99,106,107] most of which involve performing gene editing on patient cells ex vivo, enabling screening for clones containing the desired modification and without off-target modifications before transplantation back into the patient. The first clinical trial for in vivo CRISPR gene editing is currently taking place for the inherited retinal disease Leber congenital amaurosis.[107] In this trial, Cas9 and guide RNAs targeting a deep intronic variant in the *CEP290* gene are delivered by AAV vector, with the aim of excising the pathogenic intronic sequence. Since the aim is to eradicate a pathogenic pseudoexon splice site, this therapy takes advantage of the high efficiency of Cas9 for introducing indels, avoiding the need for low-efficiency HDR-mediated repair. Deep intronic variants in *USH2A* and other USH genes would also be amenable to this strategy.

SUMMARY AND PERSPECTIVE

USH2 is an incurable autosomal recessive genetic disease leading to mild to moderate congenital deafness with early-onset visual impairment. The functions of usherin are becoming increasingly revealed at the molecular level, although understanding of disease mechanisms remains incomplete. The chronology of hearing and visual impairment offers a window of opportunity for the application of molecular therapies aimed at preserving vision. If USH2 patients can receive a timely diagnosis, molecular therapies may help preserve the photoreceptors, and the development of blindness could be delayed or possibly prevented. Even when total prevention cannot be achieved, delay in disease progression can still improve the quality of life for patients. A number of promising therapies for the preservation of vision in USH patients are in development, with some currently in evaluation in clinical trials. Moreover, breakthroughs in the treatment of other inherited retinal diseases, such as Leber congenital amaurosis, are providing exciting new tools for the treatment of USH.

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Since hearing loss in USH2 patients is generally moderate and stable, clinical management with hearing aids or cochlear implants currently provides significant improvements to quality of life.[108] Preclinical evidence suggests AAV-based gene replacement therapies or AO strategies may be applicable in the inner ear as well as the eye, which could provide new and improved approaches to the treatment of hearing loss and vestibular dysfunction in USH2 patients. However, the developmental defects associated with inner ear dysfunction occur in utero in humans, and it remains unclear whether postnatal restoration of *USH2A* gene expression alone can restore hair cell architecture and function. Given USH2 hearing loss arises from a developmental defect, gene therapies for this disease may need to be combined with regenerative therapies designed to replace hair cells or reactivate inner ear development to improve hearing and vestibular function.

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