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Antisense oligonucleotides for treatment of USHER 2A. Exons 30-31

Abstract

The invention relates to the fields of medicine and immunology. In particular, it relates to novel antisense oligonucleotides that may be used in the treatment, prevention and/or delay of an USH2A related disease or condition.

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Background/Summary

FIELD OF THE INVENTION
[0001] The invention relates to the fields of medicine and immunology. In particular, it relates to novel antisense oligonucleotides that may be used in the treatment, prevention and/or delay of conditions associated with USH2A.
BACKGROUND OF THE INVENTION
[0002] Retinitis pigmentosa (RP) is a genetically and clinically heterogeneous condition that is currently still largely untreatable. Patients usually present with a progressive loss of visual function that initially manifests with night blindness and visual field constriction during adolescence, and progresses towards the loss of central vision and ultimately legal blindness in later stages of life. With a predicted overall prevalence of 1 in 4,000 individuals, RP is estimated to affect almost two million individuals worldwide. Mutations in USH2A are the most frequent cause of RP with an autosomal recessive mode of inheritance (arRP), accounting for up to 23% of all arRP cases. Besides non-syndromic RP, mutations in USH2A can also result in Usher syndrome. Patients suffering from Usher syndrome experience a double sensory impairment (combination of RP and congenital hearing impairment) making the development of a therapy to halt or delay their progressive vision loss even more urgent. The delayed onset and slowly progressive nature of USH2A-associated RP, and the nowadays often early genetic diagnosis of Usher syndrome resulting from genetic testing after the observation of congenital hearing impairment, provides ample time for therapeutic intervention.
[0003] USH2A, located on chromosome 1q41, spans approximately 800 kb and encodes two different isoforms of the usherin protein. The large usherin isoform consists of 5202 amino acids and is encoded by 72 exons. This isoform is predominantly expressed in photoreceptor cells of the retina and hair cells of the cochlea. The short isoform consists of 1546 amino acids encoded by a transcript that is built up by the 5′ 21 exons, and is expressed more widely. In total, over 600 different mutations have been identified in the transcript encoding the large isoform of usherin. As these mutations are mostly private and distributed all over the gene, the development of a mutation-independent therapy is preferred to eventually treat a significant group of patients (USH2A¬LOVD mutation database, https://databases.lovd.nl/shared/variants/USH2A/unique).
[0004] The size of the usherin-encoding sequence (15.6 kb) severely hampers the development of conventional gene augmentation therapy. The protein-encoding sequence by far exceeds the packaging capacity of adeno-associated virus (AAV) vectors (4.7 kb) and lentiviral vectors (8 kb), which are the currently preferred vehicles for retinal gene delivery. This makes conventional AAV- and LV-mediated gene augmentation therapy for USH2A-associated RP very challenging. An attractive alternative approach is antisense oligonucleotide (ASO)-induced splice modulation. In this approach, ASOs are applied to correct aberrant pre-mRNA splicing or to remove native in-frame exons harboring recurrent loss-of-function mutations. Both approaches aim to restore the original open reading frame and protein function. By targeting the pre-mRNA, ASOs are able to simultaneously and transiently modulate all endogenous transcripts encoding the different protein isoforms without altering transcription levels. We previously presented ASO-induced splice correction as a promising treatment option for the correction of aberrant mRNA splicing caused by the deep intronic c.7595-2144A>G mutation in USH2A (Slijkerman, R. W., et al, Therapy—Nucleic Acids, 2016. 5: p. e381).
[0005] More recently, we published the first ASO-based exon skipping therapy for mutations affecting USH2A exon 13 (Dulla, K., et al., Molecular Therapy, 2021). This single exon skipping therapy reached the clinical phase and resulted in a concordant benefit in multiple parameters of visual function (i.e. visual acuity, static perimetry and retinal imaging) without induction of any

serious adverse events (Trial #NCT03780257) (Therapeutics, P., *ProQR Announces Positive Results from Clinical Trial of QR*-421*a in Usher Syndrome and Plans to Start Pivotal Trials.* 2021). Skipping of USH2A exon 13 was not intended to result in the removal of a single protein domain. Instead, it resulted in the loss of 4 EGF-lam domains and formation of one EGF-like hybrid domain. The resulting shortened usherin protein was shown to retain function. The amount of single USH2A exons that meet all criteria, and of which skipping is predicted to result in a functional protein, is minimal.

[0006] However, there remains a need to for treatment options for Usher syndrome patients that are affected by mutations in, or malfunction of other regions of the transcript. To date treatment options for these Usher patients are limited to cochlear implants or hearing aids.

SUMMARY OF THE INVENTION

[0007] The invention relates to a set of antisense oligonucleotides for skipping of exon 30 and 31 that bind to and/or are complementary to a polynucleotide with the nucleotide sequence as shown in SEQ ID NO: 1. Preferably the set of antisense oligonucleotides bind to and/or are complementary to a polynucleotide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, preferably to a polynucleotide selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO:7, and SEQ ID NO: 8, and preferably to a polynucleotide selected from the group consisting of SEQ ID NO: 9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12 or a part thereof.

[0008] The invention further provides for a set of antisense oligonucleotides for skipping of exon 30 and 31 that comprises at least two antisense oligonucleotides that comprise or consist of SEQ ID NO: 13, 14, 15 or 16.

[0009] In a further aspect, the invention provides for a viral vector expressing at least one antisense oligonucleotide for skipping exons 30 or 31 as defined herein when placed under conditions conducive to expression of the molecule.

[0010] In yet a further aspect the invention provides for a pharmaceutical composition comprising the set of antisense oligonucleotides for skipping exons 30 and 31 as described herein or the viral vector as described herein and a pharmaceutically acceptable excipient.

[0011] The invention also provides for a set of antisense oligonucleotides for skipping exons 30 and 31 as described herein or the viral vector as described herein for use as a medicament. In some embodiments the set of antisense oligonucleotides for skipping exons 30 and 31 as described herein or the viral vector as described herein is for use in the treatment of a USH2A related disease or condition requiring modulating splicing of antisense oligonucleotide. In some embodiments wherein the USH2A related disease or condition is USH2A-associated Retinitis pigmentosa (RP).

[0012] The invention further provides for a method for modulating splicing of USH2A in a cell, said method comprising contacting said cell with the set of antisense oligonucleotides for skipping of exon 30 and 31 as described herein, the vector according as described herein or the pharmaceutical composition as described herein.

[0013] Lastly the invention provides for a use of the set of antisense oligonucleotides for skipping of exon 30 and 31 as described herein, the vector as described herein or the pharmaceutical composition according to as described herein for treating an USH2A related disease or a condition requiring modulating splicing of USH2A.

DETAILED DESCRIPTION OF THE INVENTION

[0014] Calculations based on available carrier frequencies in the general population for the various reported pathogenic variants indicate that 22,000 individuals worldwide suffer from USH2A-associated disease caused by (a) variant(s) in these exons. Surprisingly it has now been demonstrated that specific antisense oligonucleotides (ASOs) are able to induce the dual skipping of exons 30 and 31. This dual exon skipping results in a transcript encoding a shortened usherin protein that lacks exactly one of the repetitive fibronectin type III (FN3) domains. This carefully designed dual exon skipping approach is further shown to be highly promising as a future treatment

option for USH2A-associated RP.
[0015] Accordingly, in a first aspect, the invention relates to a set of antisense oligonucleotides for the skipping of exon 30 and 31 of USH2A that bind to and/or are complementary to a polynucleotide with the nucleotide sequence as shown in SEQ ID NO: 1. Preferably the antisense oligonucleotides bind to and/or are complementary to a polynucleotide with the nucleotide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4. More preferably the polynucleotide is selected from the group consisting of SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO: 8 and even more preferably the polynucleotide is selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO:11 and SEQ ID NO:12 or a part thereof.
[0016] The terms “antisense oligonucleotide”, “ASO” and “AON” are used interchangeably herein and are understood to refer to an oligonucleotide molecule comprising a nucleotide sequence which is substantially complementary to a target nucleotide sequence in a pre-mRNA molecule, hnRNA (heterogenous nuclear RNA) or mRNA molecule. The degree of complementarity (or substantial complementarity) of the antisense sequence is preferably such that a molecule comprising the antisense sequence can form a stable hybrid with the target nucleotide sequence in the RNA molecule under physiological conditions. Binding of an ASO to its target can easily be assessed by the person skilled in the art using techniques that are known in the field such as the gel mobility shift assay as described in EP1619249.
[0017] The term “set” as used herein is defined as comprising at least two, at least three, at least four or more antisense oligonucleotides that bind and/or are complementary to a polynucleotide with the nucleotide sequence as shown in SEQ ID NO: 1, preferably selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, preferably selected from the group consisting of SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO: 8, preferably selected from the group consisting of SEQ ID NO: 9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12 or a part thereof. In certain embodiments the set of antisense oligonucleotides according to the invention comprises at least one antisense oligonucleotide that binds and/or is complementary to SEQ ID NO: 3, preferably that binds and/or is complementary to SEQ ID NO: 5 and SEQ ID NO:6, preferably selected from the group consisting of SEQ ID NO: 9 and SEQ ID NO:10 or a part thereof and at least one antisense oligonucleotide that binds and/or is complementary to SEQ ID NO: 4, preferably that binds and/or is complementary to SEQ ID NO: 7 and SEQ ID NO:8, preferably selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO:12 or a part thereof. In certain embodiments the set of antisense oligonucleotides according to the invention comprises at least two antisense oligonucleotides that binds to SEQ ID NO: 3, preferably that binds and/or is complementary to SEQ ID NO: 5 and SEQ ID NO:6, preferably selected from the group consisting of SEQ ID NO: 9 and SEQ ID NO:10 or a part thereof and at least one antisense oligonucleotide that binds to SEQ ID NO: 4 preferably that binds and/or is complementary to SEQ ID NO: 7 and SEQ ID NO:8, preferably selected from the group consisting of SEQ ID NO: 11 and SEQ ID NO:12 or a part thereof.
[0018] The term “complementary” used in the context of the invention indicates that some mismatches in the antisense sequence are allowed as long as the functionality, i.e. inducing the skipping of exons 30-31 is achieved. Preferably, the complementarity is from 90% to 100%. In general this allows for 1 or 2 mismatches in an ASO of 20 nucleotides or 1, 2, 3 or 4 mismatches in an ASO of 40 nucleotides, or 1, 2, 3, 4, 5 or 6 mismatches in an ASO of 60 nucleotides, etc. Optionally, said ASO may further be tested by transfection into isolated cells comprising USH2A. The complementary regions are preferably designed such that, when combined, they are specific for the intron or exon in the pre-mRNA or mRNA. Such specificity may be created with various lengths of complementary regions, as this depends on the actual sequences in other (pre-) mRNA molecules in the system. The risk that the ASO will also be able to hybridize to one or more other (pre-) mRNA molecules decreases with increasing size of the ASO. It is clear that ASOs comprising mismatches in the region of complementarity but that retain the capacity to hybridize

and/or bind to the targeted region(s) in the (pre-) mRNA, can be used in the invention. However, preferably at least the complementary parts do not comprise such mismatches as ASOs lacking mismatches in the complementary part typically have a higher efficiency and a higher specificity than ASOs having such mismatches in one or more complementary regions. It is thought, that higher hybridization strengths, (i.e. increasing number of interactions with the opposing strand) are favorable in increasing the efficiency of the process of interfering with the splicing or mRNA degradation machinery of the system.

[0019] Each ASO withing the set ASOs of according to the invention preferably does not contain a stretch of CpG, more preferably does not contain any CpG. The presence of a CpG or a stretch of CpG in an oligonucleotide is usually associated with an increased immunogenicity of said oligonucleotide (Dorn and Kippenberger, 2008). This increased immunogenicity is undesired since it may induce damage of the tissue to be treated, i.e. the retina or the inner ear. Immunogenicity may be assessed in an animal model by assessing the presence of CD4+ and/or CD8+ cells and/or inflammatory mononucleocyte infiltration. Immunogenicity may also be assessed in blood of an animal or of a human being treated with an ASO according to the invention by detecting the presence of a neutralizing antibody and/or an antibody recognizing said ASO using a standard immunoassay known to the skilled person. An inflammatory reaction, type I-like interferon production, IL-12 production and/or an increase in immunogenicity may be assessed by detecting the presence or an increasing amount of a neutralizing antibody or an antibody recognizing said ASO using a standard immunoassay. The ASO according to the invention furthermore preferably has acceptable RNA binding kinetics and/or thermodynamic properties. The RNA binding kinetics and/or thermodynamic properties are at least in part determined by the melting temperature of an oligonucleotide (Tm; calculated with the oligonucleotide properties calculator (www.unc.edu/-cail/biotool/oligo/index) for single stranded RNA using the basic Tm and the nearest neighbor model), and/or the free energy of the ASO-target intron/exon complex (using RNA structure version 4.5). If a Tm is too high, the ASO is expected to be less specific. An acceptable Tm and free energy depend on the sequence of the ASO. Therefore, it is difficult to give preferred ranges for each of these parameters. An acceptable Tm may be ranged between 35 and 70° C. and an acceptable free energy may be ranged between 15 and 45 kcal/mol.

[0020] In certain embodiments, each of the antisense oligonucleotides within the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention has a length of from about 8 to about 40 nucleotides, preferably from about 10 to about 40 nucleotides, more preferably from about 14 to about 30 nucleotides, more preferably from about 16 to about 24 nucleotides, such as 16, 17, 18, 19, 20, 21, 22, 23 or 24 nucleotides.

[0021] In certain embodiments, the set of antisense oligonucleotides for skipping of exon 30 and 31 according as described herein comprises at least two antisense oligonucleotides that comprise or consist of SEQ ID NO: 13, 14, 15 or 16. In certain embodiments, the set of antisense oligonucleotides for skipping of exon 30 and 31 according as described herein comprises at least three or at least four antisense oligonucleotides that comprise or consist of SEQ ID NO: 13, 14, 15 or 16.

[0022] In certain embodiments, the set of antisense oligonucleotides according to the invention is selected from the group consisting of: [0023] an antisense oligonucleotides that comprises or consists of SEQ ID NO: 13 and an antisense oligonucleotides that comprises or consists of SEQ ID NO: 15; [0024] an antisense oligonucleotides that comprises or consists of SEQ ID NO: 13 and an antisense oligonucleotides that comprises or consists of SEQ ID NO: 16; [0025] an antisense oligonucleotides that comprises or consists of SEQ ID NO: 14 and an antisense oligonucleotides that comprises or consists of SEQ ID NO: 15; and [0026] an antisense oligonucleotides that comprises or consists of SEQ ID NO: 14 and an antisense oligonucleotides that comprises or consists of SEQ ID NO: 16.

[0027] In certain embodiments, the set of antisense oligonucleotides according to the invention is

selected from the group consisting of: [0028] an antisense oligonucleotides that comprises or consists of SEQ ID NO: 15, an antisense oligonucleotides that comprises or consists of SEQ ID NO: 13 and an antisense oligonucleotides that comprises or consists of SEQ ID NO: 14; and [0029] an antisense oligonucleotides that comprises or consists of SEQ ID NO: 16, an antisense oligonucleotides that comprises or consists of SEQ ID NO: 13 and an antisense oligonucleotides that comprises or consists of SEQ ID NO: 14.

[0030] It is preferred that each antisense oligonucleotide for skipping exons 30 and 31 within the set of antisense oligonucleotides of the invention comprises one or more residues that are modified to increase nuclease resistance, and/or to increase the affinity of the antisense oligonucleotide for the target sequence. Therefore, in a certain embodiment, the antisense nucleotide sequence comprises at least one nucleotide analogue or equivalent, wherein a nucleotide analogue or equivalent is defined as a residue having a modified base, and/or a modified backbone, and/or a non-natural internucleoside linkage, or a combination of these modifications.

[0031] In certain embodiments, the nucleotide analogue or equivalent comprises a modified backbone. Examples of such backbones are provided by morpholino backbones, carbamate backbones, siloxane backbones, sulfide, sulfoxide and sulfone backbones, formacetyl and thioformacetyl backbones, methyleneformacetyl backbones, riboacetyl backbones, alkene containing backbones, sulfamate, sulfonate and sulfonamide backbones, methyleneimino and methylenehydrazino backbones, and amide backbones. Phosphorodiamidate morpholino oligomers are modified backbone oligonucleotides that have previously been investigated as antisense agents.

[0032] Morpholino oligonucleotides have an uncharged backbone in which the deoxyribose sugar of DNA is replaced by a six membered ring and the phosphodiester linkage is replaced by a phosphorodiamidate linkage. Morpholino oligonucleotides are resistant to enzymatic degradation and appear to function as antisense agents by arresting translation or interfering with pre-mRNA splicing rather than by activating RNase H. Morpholino oligonucleotides have been successfully delivered to tissue culture cells by methods that physically disrupt the cell membrane, and one study comparing several of these methods found that scrape loading was the most efficient method of delivery; however, because the morpholino backbone is uncharged, cationic lipids are not effective mediators of morpholino oligonucleotide uptake in cells. A recent report demonstrated triplex formation by a morpholino oligonucleotide and, because of the non-ionic backbone, these studies showed that the morpholino oligonucleotide was capable of triplex formation in the absence of magnesium.

[0033] In further embodiments that the linkage between the residues in a backbone do not include a phosphorus atom, such as a linkage that is formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages.

[0034] Examples of nucleotide analogue or equivalent comprises a Peptide Nucleic Acid (PNA), having a modified polyamide backbone (Nielsen, et al. (1991) Science 254, 1497-1500). PNA-based molecules are true mimics of DNA molecules in terms of base-pair recognition. The backbone of the PNA is composed of N-(2-aminoethyl)-glycine units linked by peptide bonds, wherein the nucleobases are linked to the backbone by methylene carbonyl bonds. An alternative backbone comprises a one-carbon extended pyrrolidine PNA monomer (Govindaraju and Kumar (2005) Chem. Commun, 495-497). Since the backbone of a PNA molecule contains no charged phosphate groups, PNA-RNA hybrids are usually more stable than RNA-RNA or RNA-DNA hybrids, respectively (Egholm et al (1993) Nature 365, 566-568). In certain embodiments the backbone comprises a morpholino nucleotide analog or equivalent, in which the ribose or deoxyribose sugar is replaced by a 6-membered morpholino ring. In certain embodiments the nucleotide analog or equivalent comprises a phosphorodiamidate morpholino oligomer (PMO), in which the ribose or deoxyribose sugar is replaced by a 6-membered morpholino ring, and the anionic phosphodiester linkage between adjacent morpholino rings is replaced by a non-ionic

phosphorodiamidate linkage.
[0035] In yet a further embodiment, a nucleotide analogue or equivalent of the invention comprises a substitution of one of the non-bridging oxygens in the phosphodiester linkage. This modification slightly destabilizes base-pairing but adds significant resistance to nuclease degradation. A preferred nucleotide analogue or equivalent comprises phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, H-phosphonate, methyl and other alkyl phosphonate including 3′-alkylene phosphonate, 5′-alkylene phosphonate and chiral phosphonate, phosphinate, phosphoramidate including 3′-amino phosphoramidate and aminoalkylphosphoramidate, thionophosphoramidate, thionoalkylphosphonate, thionoalkylphosphotriester, selenophosphate or boranophosphate.
[0036] In certain embodiments the nucleotide analogue or equivalent of the invention comprises one or more sugar moieties that are mono- or disubstituted at the 2′, 3′ and/or 5′ position such as a —OH; —F; substituted or unsubstituted, linear or branched lower (Cl—C10) alkyl, alkenyl, alkynyl, alkaryl, allyl, or aralkyl, that may be interrupted by one or more heteroatoms; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; O-, S-, or N-allyl; O-alkyl-O-alkyl, -methoxy, -aminopropoxy; methoxyethoxy; dimethylaminooxyethoxy; and -dimethylaminoethoxyethoxy.
[0037] The sugar moiety can be a pyranose or derivative thereof, or a deoxypyranose or derivative thereof, preferably ribose or derivative thereof, or deoxyribose or derivative of. A preferred derivatized sugar moiety comprises a Locked Nucleic Acid (LNA), in which the 2′-carbon atom is linked to the 3′ or 4′ carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. A preferred LNA comprises 2′-O, 4′-C-ethylene-bridged nucleic acid (Morita et al. 2001. Nucleic Acid Res Supplement No. 1:241-242). These substitutions render the nucleotide analogue or equivalent RNase H and nuclease resistant and increase the affinity for the target RNA.
[0038] In another embodiment, a nucleotide analogue or equivalent of the invention comprises one or more base modifications or substitutions. Modified bases comprise synthetic and natural bases such as inosine, xanthine, hypoxanthine and other -aza, deaza, -hydroxy, -halo, -thio, thiol, -alkyl, -alkenyl, -alkynyl, thioalkyl derivatives of pyrimidine and purine bases that are or will be known in the art.
[0039] It is understood by a skilled person that it is not necessary for all positions in an antisense oligonucleotide to be modified uniformly. In addition, more than one of the aforementioned analogues or equivalents may be incorporated in a single antisense oligonucleotide or even at a single position within an antisense oligonucleotide. In certain embodiments, an antisense oligonucleotide of the invention has at least two different types of analogues or equivalents.
[0040] Accordingly, each antisense oligonucleotide for skipping exons 30 and 31 within the set of antisense oligonucleotides of the invention comprises a 2′-O alkyl phosphorothioate antisense oligonucleotide, such as 2′-O-methyl modified ribose (RNA), 2′-O-ethyl modified ribose, 2′-O-methoxyethyl modified ribose, 2′-O-propyl modified ribose, and/or substituted derivatives of these modifications such as halogenated derivatives.
[0041] In certain embodiments, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention comprises an antisense oligonucleotide that comprises or consists of SEQ ID NO: 13 that comprises a 2′-O-methoxyethyl modified ribose and a phosphorothioate backbone.
[0042] In certain embodiments, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention comprises an antisense oligonucleotide that comprises or consists of SEQ ID NO: 14 that comprises a 2′-O-methoxyethyl modified ribose and a phosphorothioate backbone.
[0043] In certain embodiments, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention comprises an antisense oligonucleotide that comprises or consists of SEQ ID NO: 15 that comprises a 2′-O-methoxyethyl modified ribose and a phosphorothioate backbone.

[0044] In certain embodiments, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention comprises an antisense oligonucleotide comprises or consists of SEQ ID NO: 16 that comprises a 2′-O-methoxyethyl modified ribose and a phosphorothioate backbone.
[0045] Each antisense oligonucleotide for skipping exons 30 and 31 according to the invention, preferably, may be delivered as such. However, an antisense oligonucleotide for skipping exons 30 and 31 may also be encoded by a viral vector. Typically, this is in the form of an RNA transcript that comprises the sequence of an oligonucleotide according to the invention in a part of the transcript. Accordingly, in one aspect, the invention provides for a viral vector expressing at least one antisense oligonucleotide for skipping exons 30 or 31 as defined herein when placed under conditions conducive to expression of the molecule.
[0046] Viral vectors as used herein include but are not limited to lentiviral vector systems and adenoviral vector systems.
[0047] A preferred expression system for an ASO for skipping exons 30 and 31 according to the invention is an adenovirus associated virus (AAV)-based vector. Single chain and double chain AAV-based vectors have been developed that can be used for prolonged expression of antisense nucleotide sequences for highly efficient degradation of transcripts. A preferred AAV-based vector, for instance, comprises an expression cassette that is driven by an RNA polymerase Ill-promoter (Pol III) or an RNA polymerase II promoter (Pol II). A preferred RNA promoter is, for example, a Pol III U6 RNA promoter, or a Pol II U7 RNA promoter.
[0048] The invention accordingly provides for a viral-based vector, comprising a Pol II or a Pol III promoter driven expression cassette for expression of an antisense oligonucleotide for skipping exons 30-31 of USHA2A.
[0049] An AAV vector according to the invention is a recombinant AAV vector and refers to an AAV vector comprising part of an AAV genome comprising an encoded ASO for the skipping of exons 30-31 of USH2A according to the invention encapsidated in a protein shell of capsid protein derived from an AAV serotype as depicted elsewhere herein. Part of an AAV genome may contain the inverted terminal repeats (ITR) derived from an adeno-associated virus serotype, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV8, AAV9 and others. A protein shell comprised of capsid protein may be derived from an AAV serotype such as AAV1, 2, 3, 4, 5, 8, 9 and others. A protein shell may also be named a capsid protein shell. AAV vector may have one or preferably all wild type AAV genes deleted, but may still comprise functional ITR nucleic acid sequences. Functional ITR sequences are necessary for the replication, rescue and packaging of AAV virions. The ITR sequences may be wild type sequences or may have at least 80%, 85%, 90%, 95, or 100% sequence identity with wild type sequences or may be altered by for example in insertion, mutation, deletion or substitution of nucleotides, as long as they remain functional. In this context, functionality refers to the ability to direct packaging of the genome into the capsid shell and then allow for expression in the host cell to be infected or target cell. In the context of the invention a capsid protein shell may be of a different serotype than the AAV vector genome ITR. An AAV vector according to present the invention may thus be composed of a capsid protein shell, i.e. the icosahedral capsid, which comprises capsid proteins (VP1, VP2, and/or VP3) of one AAV serotype, e.g. AAV serotype 2, whereas the ITRs sequences contained in that AAV5 vector may be any of the AAV serotypes described above, including an AAV2 vector. An “AAV2 vector” thus comprises a capsid protein shell of AAV serotype 2, while e.g. an “AAV5 vector” comprises a capsid protein shell of AAV serotype 5, whereby either may encapsidate any AAV vector genome ITR according to the invention.
[0050] Preferably, a recombinant AAV vector according to the invention comprises a capsid protein shell of AAV serotype 2, 5, 8 or AAV serotype 9 wherein the AAV genome or ITRs present in said AAV vector are derived from AAV serotype 2, 5, 8 or AAV serotype 9; such AAV vector is referred to as an AAV2/2, AAV 2/5, AAV2/8, AAV2/9, AAV5/2, AAV5/5, AAV5/8, AAV 5/9, AAV8/2, AAV 8/5, AAV8/8, AAV8/9, AAV9/2, AAV9/5, AAV9/8, or an AAV9/9 vector.

[0051] More preferably, a recombinant AAV vector according to the invention comprises a capsid protein shell of AAV serotype 2 and the AAV genome or ITRs present in said vector are derived from AAV serotype 5; such vector is referred to as an AAV 2/5 vector.
[0052] More preferably, a recombinant AAV vector according to the invention comprises a capsid protein shell of AAV serotype 2 and the AAV genome or ITRs present in said vector are derived from AAV serotype 8; such vector is referred to as an AAV 2/8 vector.
[0053] More preferably, a recombinant AAV vector according to the invention comprises a capsid protein shell of AAV serotype 2 and the AAV genome or ITRs present in said vector are derived from AAV serotype 9; such vector is referred to as an AAV 2/9 vector.
[0054] More preferably, a recombinant AAV vector according to the invention comprises a capsid protein shell of AAV serotype 2 and the AAV genome or ITRs present in said vector are derived from AAV serotype 2; such vector is referred to as an AAV 2/2 vector.
[0055] A nucleic acid molecule encoding an ASO according to the invention represented by a nucleic acid sequence of choice is preferably inserted between the AAV genome or ITR sequences as identified above, for example an expression construct comprising an expression regulatory element operably linked to a coding sequence and a 3′ termination sequence.
[0056] “AAV helper functions” generally refers to the corresponding AAV functions required for AAV replication and packaging supplied to the AAV vector in trans. AAV helper functions complement the AAV functions which are missing in the AAV vector, but they lack AAV ITRs (which are provided by the AAV vector genome). AAV helper functions include the two major ORFs of AAV, namely the rep coding region and the cap coding region or functional substantially identical sequences thereof. Rep and Cap regions are well known in the art, see e.g. (Chiorini et al., 1999) or U.S. Pat. No. 5,139,941, incorporated herein by reference. The AAV helper functions can be supplied on an AAV helper construct, which may be a plasmid. Introduction of the helper construct into the host cell can occur e.g. by transformation, transfection, or transduction prior to or concurrently with the introduction of the AAV genome present in the AAV vector as identified herein. The AAV helper constructs according to the invention may thus be chosen such that they produce the desired combination of serotypes for the AAV vector's capsid protein shell on the one hand and for the AAV genome present in said AAV vector replication and packaging on the other hand.
[0057] “AAV helper virus” provides additional functions required for AAV replication and packaging. Suitable AAV helper viruses include adenoviruses, herpes simplex viruses (such as HSV types 1 and 2) and vaccinia viruses. The additional functions provided by the helper virus can also be introduced into the host cell via vectors, as described in U.S. Pat. No. 6,531,456 incorporated herein by reference.
[0058] Preferably, an AAV genome as present in a recombinant AAV vector according to the invention does not comprise any nucleotide sequences encoding viral proteins, such as the rep (replication) or cap (capsid) genes of AAV. An AAV genome may further comprise a marker or reporter gene, such as a gene for example encoding an antibiotic resistance gene, a fluorescent protein (e.g. gfp) or a gene encoding a chemically, enzymatically or otherwise detectable and/or selectable product (e.g. lacZ, aph, etc.) known in the art.
[0059] A preferred AAV vector according to the invention is an AAV vector, preferably an AAV2/5, AAV2/8, AAV2/9 or AAV2/2 vector, carrying an ASO for skipping exons 30 and 31 according to the invention that is an ASO that comprises, or preferably consists of, a sequence that is: [0060] complementary or substantially complementary to a nucleotide sequence consisting of SEQ ID NO 1, preferably selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, preferably selected from the group consisting of SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO: 7, and SEQ ID NO: 8, preferably selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO:12 or a part thereof.
[0061] Even more preferably, the ASO comprises or consists of a polynucleotide with a nucleotide

sequence selected from the group consisting of SEQ ID NO: 13, 14, 15 and 16.
[0062] Improvements in means for providing an individual or a cell, tissue, organ of said individual with an antisense oligonucleotide for skipping exons 30-31 according to the invention, are anticipated considering the progress that has already thus far been achieved. Such future improvements may of course be incorporated to achieve the mentioned effect on restructuring of mRNA using a method according to the invention.
[0063] Alternatively, a preferred delivery method for an antisense oligonucleotide for skipping exons 30-31 as described herein or a plasmid for expression of such ASO is a viral vector or are nanoparticles. In certain embodiments, the preferred delivery method for an ASO as described herein is by use of slow-release or sustained release capsules. In certain embodiments, the preferred delivery method for an ASO as described herein is by use of hydrogels (such as described in WO1993/01286).
[0064] Alternatively, a preferred delivery method for an antisense oligonucleotide or a plasmid for antisense oligonucleotide expression is a viral vector or nanoparticles. Preferably viral vectors or nanoparticles are delivered to retina or inner ear cells. Such delivery to retina or inner ear cells or other relevant cells may be in vivo, in vitro or ex vivo.
[0065] Alternatively, a plasmid can be provided by transfection using known transfection agentia. For intravenous, subcutaneous, intramuscular, intrathecal and/or intraventricular administration it is preferred that the solution is a physiological salt solution. Particularly preferred in the invention is the use of an excipient or transfection agentia that will aid in delivery of each of the constituents as defined herein to a cell and/or into a cell, preferably a retina cell. Preferred are excipients or transfection agentia capable of forming complexes, nanoparticles, micelles, vesicles and/or liposomes that deliver each constituent as defined herein, complexed or trapped in a vesicle or liposome through a cell membrane. Many of these excipients are known in the art. Suitable excipients or transfection agentia comprise polyethylenimine (PEI; ExGen500 (MBI Fermentas)), LipofectAMINE™ 2000 (Invitrogen) or derivatives thereof, or similar cationic polymers, including polypropyleneimine or polyethylenimine copolymers (PECs) and derivatives, synthetic amphiphils (SAINT-18), Lipofectin™, DOTAP and/or viral capsid proteins that are capable of self assembly into particles that can deliver each constitutent as defined herein to a cell, preferably a retina cell. Such excipients have been shown to efficiently deliver an oligonucleotide such as antisense nucleic acids to a wide variety of cultured cells, including retina cells. Their high transfection potential is combined with an excepted low to moderate toxicity in terms of overall cell survival. The ease of structural modification can be used to allow further modifications and the analysis of their further (in vivo) nucleic acid transfer characteristics and toxicity.
[0066] Lipofectin represents an example of a liposomal transfection agent. It consists of two lipid components, a cationic lipid N-[1-(2,3 dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA) (cp. DOTAP which is the methylsulfate d a neutral lipid dioleoylphosphatidylethanolamine (DOPE). The neutral component mediates the intracellular release. Another group of delivery systems are polymeric nanoparticles.
[0067] Polycations such as diethylaminoethylaminoethyl (DEAE)-dextran, which are well known as DNA transfection reagent can be combined with butylcyanoacrylate (PBCA) and hexylcyanoacrylate (PHCA) to formulate cationic nanoparticles that can deliver each constituent as defined herein, preferably an oligonucleotide, across cell membranes into cells.
[0068] In addition to these common nanoparticle materials, the cationic peptide protamine offers an alternative approach to formulate an oligonucleotide with colloids. This colloidal nanoparticle system can form so called proticles, which can be prepared by a simple self-assembly process to package and mediate intracellular release of an oligonucleotide. The skilled person may select and adapt any of the above or other commercially available alternative excipients and delivery systems to package and deliver an exon skipping molecule for use in the current invention to deliver it for the prevention, treatment or delay of a USH2A related disease or condition. “Prevention, treatment

or delay of a USH2A related disease or condition" is herein preferably defined as preventing, halting, ceasing the progression of, or reversing partial or complete visual impairment or blindness, as well as preventing, halting, ceasing the progression of or reversing partial or complete auditory impairment or deafness that is caused by a genetic defect in the USH2A gene.

[0069] An antisense oligonucleotide can be linked to a moiety that enhances uptake of the antisense oligonucleotide in cells, preferably retina cells. Examples of such moieties are cholesterols, carbohydrates, vitamins, biotin, lipids, phospholipids, cell-penetrating peptides including but not limited to antennapedia, TAT, transportan and positively charged amino acids such as oligoarginine, poly-arginine, oligolysine or polylysine, antigen-binding domains such as provided by an antibody, a Fab fragment of an antibody, or a single chain antigen binding domain such as a cameloid single domain antigen-binding domain.

[0070] In addition, each antisense oligonucleotide within the set of antisense oligonucleotides according to the invention could be covalently or non-covalently linked to a targeting ligand specifically designed to facilitate the uptake into the cell, cytoplasm and/or its nucleus. Such ligand could comprise (i) a compound (including but not limited to peptide(-like) structures) recognising cell, tissue or organ specific elements facilitating cellular uptake and/or (ii) a chemical compound able to facilitate the uptake in to cells and/or the intracellular release of an oligonucleotide from vesicles, e.g. endosomes or lysosomes.

[0071] Therefore, in a preferred embodiment, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention according to the invention is formulated in a composition or a medicament or a composition, which is provided with at least an excipient and/or a targeting ligand for delivery and/or a delivery device thereof to a cell and/or enhancing its intracellular delivery.

[0072] It is to be understood that if a composition comprises an additional constituent such as an adjunct compound as later defined herein, each constituent of the composition may not be formulated in one single combination or composition or preparation. Depending on their identity, the skilled person will know which type of formulation is the most appropriate for each constituent as defined herein. In a preferred embodiment, the invention provides a composition or a preparation which is in the form of a kit of parts comprising an exon skipping molecule according to the invention and a further adjunct compound as later defined herein.

[0073] If required, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention or the vector, preferably viral vector of the invention, can be incorporated into a pharmaceutically active mixture by adding a pharmaceutically acceptable carrier.

[0074] Accordingly, the invention also provides a composition, preferably a pharmaceutical composition, comprising the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention and a pharmaceutically acceptable excipient. Such composition may comprise at least two, at least three or at least four antisense oligonucleotides, or at least two, at least three or at least four viral vectors according to the invention. Such a pharmaceutical composition may comprise any pharmaceutically acceptable excipient, including a carrier, filler, preservative, adjuvant, solubilizer and/or diluent. Such pharmaceutically acceptable carrier, filler, preservative, adjuvant, solubilizer and/or diluent may for instance be found in Remington, 2000. Each feature of said composition has earlier been defined herein.

[0075] In certain embodiments, the pharmaceutical composition as described herein is administered through intravitreal or intratympanic administration. For delivery to the brain or pineal gland intrathecal injections or systemic delivery is also considered. Concentration or dose defined herein may refer to the total concentration or dose of all oligonucleotides used or the concentration or dose of each exon skipping molecule used or added. Therefore in one embodiment, there is provided a composition wherein each or the total amount of antisense oligonucleotides according to the invention used is dosed in an amount ranged from 0.01 and 30 mg/kg, preferably from 0.05 and 30

mg/kg. A suitable intravitreal dose would be between 0.05 mg and 5 mg, preferably between 0.1 and 1 mg per eye, such as about per eye: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 mg. A suitable in intratympanic dose would be between 0.1 mg and 30 mg, preferably between 0.1 and 15 mg per ear, such as about: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0, or 15.0 mg per ear.

[0076] Preferably, the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention, the vector as described herein and the pharmaceutical composition as described herein is for use in the treatment of a USH2A related disease or condition of an individual. In all embodiments of the invention, the term “treatment” is understood to include the prevention and/or delay of the USH2A-related disease or condition. An individual, which may be treated using an antisense oligonucleotide for skipping exons 30-31 according to the invention, the vector as described herein and the pharmaceutical composition as described herein may already have been diagnosed as having a USH2A-related disease or condition.

[0077] Alternatively, an individual which may be treated using the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention may not have yet been diagnosed as having a USH2A-related disease or condition but may be an individual having an increased risk of developing a USH2A-related disease or condition in the future given his or her genetic background. A preferred individual is a human being. In a preferred embodiment the USH2A-related disease or condition is Usher Syndrome type 2.

[0078] Accordingly, the invention further provides a set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention, or a composition according to the invention for use as a medicament, for treating a USH2A-related disease or condition requiring modulating splicing of USH2A and for use as a medicament for the prevention, treatment or delay of a USH2A-related disease or condition. A preferred USH2A-related disease or condition is Usher Syndrome type 2.

[0079] The invention further provides the use of a set of antisense oligonucleotide for skipping exons 30 and 31 according to the invention, or of a viral vector according to the invention, or a composition according to the invention for the treatment of a USH2A-related disease or condition requiring modulating splicing of USH2A. In a preferred embodiment the USH2A-related disease or condition is USH2A-associated Retinitis pigmentosa (RP).

[0080] The invention further provides the use of a set of antisense oligonucleotide for skipping exons 30 and 31 according to the invention, or of a viral vector according to the invention, or a composition according to the invention for the preparation of a medicament, for the preparation of a medicament for treating a USH2A-related disease or condition requiring modulating splicing of USH2A and for the preparation of a medicament for the prevention, treatment or delay of a USH2A-related disease or condition. A preferred USH2A-related disease or condition is Usher Syndrome type 2. Therefore in a further aspect, there is provided the use of a set of antisense oligonucleotide for skipping exons 30 and 31, viral vector or composition as defined herein for the preparation of a medicament, for the preparation of a medicament for treating a condition requiring modulating splicing of USH2A and for the preparation of a medicament for the prevention, treatment or delay of a USH2A-related disease or condition. A preferred USH2A-related disease or condition is USH2A-associated Retinitis pigmentosa (RP).

[0081] A treatment in a use or in a method according to the invention is at least once, lasts one week, one month, several months, one year, 2, 3, 4, 5, 6 years or longer, such as lifelong. Each antisense oligonucleotide for skipping exons 30 and 31 or equivalent thereof as defined herein for use according to the invention may be suitable for direct administration to a cell, tissue and/or an organ in vivo of individuals already affected or at risk of developing USH2A-related disease or condition, and may be administered directly in vivo, ex vivo or in vitro. The frequency of administration of an oligonucleotide, composition, compound or adjunct compound of the invention may depend on several parameters such as the severity of the disease, the age of the

patient, the mutation of the patient, the number of antisense oligonucleotides (i.e. dose), the formulation of antisense oligonucleotides, the route of administration and so forth. The frequency may vary between daily, weekly, at least once in two weeks, or three weeks or four weeks or five weeks or a longer time period.

[0082] Dose ranges of oligonucleotides according to the invention are preferably designed on the basis of rising dose studies in clinical trials (in vivo use) for which rigorous protocol requirements exist. An oligonucleotide as defined herein, may be used at a dose which is ranged from 0.01 and 20 mg/kg, preferably from 0.05 and 20 mg/kg. A suitable intravitreal or intratympanic dose would be between 0.05 mg and 5 mg, preferably between 0.1 and 1 mg per eye or per ear, such as about per eye: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 mg.

[0083] In a preferred embodiment, a concentration of an oligonucleotide as defined herein, which is ranged from 0.1 nM and 1 μM is used. Preferably, this range is for in vitro use in a cellular model such as retina or cochlear cells or retinal or cochlear tissue. More preferably, the concentration used is ranged from 1 to 400 nM, even more preferably from 10 to 200 nM, even more preferably from 50 to 100 nM. If several oligonucleotides are used, this concentration or dose may refer to the total concentration or dose of oligonucleotides or the concentration or dose of each oligonucleotide added.

[0084] In a preferred embodiment, a viral vector, preferably an AAV vector as described earlier herein, as delivery vehicle for a oligonucleotide according to the invention, is administered in a dose ranging from 1×10.sup.9-1×10.sup.17 virus particles per injection, more preferably from 1×10.sup.10-1×10.sup.12 virus particles per injection.

[0085] The ranges of concentration or dose of oligonucleotide(s) as given above are preferred concentrations or doses for in vivo, in vitro or ex vivo uses. The skilled person will understand that depending on the oligonucleotide(s) used, the target cell to be treated, the gene target and its expression levels, the medium used and the transfection and incubation conditions, the concentration or dose of oligonucleotide(s) used may further vary and may need to be optimized any further.

[0086] The set of antisense oligonucleotide for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention, or a composition according to the invention for use according to the invention may be suitable for administration to a cell, tissue and/or an organ in vivo of individuals already affected or at risk of developing a USH2A-related disease or condition, and may be administered in vivo, ex vivo or in vitro. The set of antisense oligonucleotide for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention, or a composition according to the invention may be directly or indirectly administered to a cell, tissue and/or an organ in vivo of an individual already affected by or at risk of developing a USH2A-related disease or condition, and may be administered directly or indirectly in vivo, ex vivo or in vitro. As Usher Syndrome type 2 has a pronounced phenotype in retina and inner ear cells, it is preferred that said cells are retina or inner ear cells, it is further preferred that said tissue is the retina or the inner ear and/or it is further preferred that said organ comprises or consists of the eye or the ear.

[0087] The invention further provides a method for modulating splicing of USH2A in a cell comprising contacting the cell, preferably a retina cell, with a set of antisense oligonucleotide for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention, or a composition according to the invention. The features of this aspect are preferably those defined earlier herein. Contacting the cell with an exon skipping molecule according to the invention, or a viral vector according to the invention, or a composition according to the invention may be performed by any method known by the person skilled in the art. Use of the methods for delivery of the set of antisense oligonucleotide for skipping exons 30 and 31, viral vectors and compositions described herein is included. Contacting may be directly or indirectly and may be in vivo, ex vivo or in vitro.

[0088] The invention further provides a method for the treatment of a USH2A-related disease or condition requiring modulating splicing of USH2A of an individual in need thereof, said method comprising contacting a cell, preferably a retina cell or cochlear cell, of said individual with a set of antisense oligonucleotide for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention, or a composition according to the invention. The features of this aspect are preferably those defined earlier herein. Contacting the cell, preferably a retina cell or a cochlear cell with a set of oligonucleotides according to the invention, or a viral vector according to the invention, or a composition according to the invention may be performed by any method known by the person skilled in the art. Use of the methods for delivery of molecules, viral vectors and compositions described herein is included. Contacting may be directly or indirectly and may be in vivo, ex vivo or in vitro.
[0089] In yet another aspect, the invention provides for the use of the set of antisense oligonucleotides for skipping exons 30 and 31 according to the invention, or a viral vector according to the invention, or a composition according to the invention for treating an USH2A-related disease or a condition requiring modulating splicing of USH2A.
[0090] Unless otherwise indicated each embodiment as described herein may be combined with another embodiment as described herein.
[0091] In this document and in its claims, the verb “to comprise” and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article “a” or “an” does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article “a” or “an” thus usually means “at least one”.
[0092] The word “about” or “approximately” when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 5% of the value.
[0093] The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The skilled person is capable of identifying such erroneously identified bases and knows how to correct for such errors.
[0094] All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

Description

DESCRIPTION OF THE FIGURES
[0095] FIG. **1**: Overview of target sites for the designed antisense oligonucleotides (ASOs). ASOs that specifically induce skipping of (A) USH2A exon 30 or (B) USH2A exon 31 were designed. ASOs target either the intron-exon boundaries, or exonic splicing enhancer (ESE) motifs within the exons. Splicing Enhancer Matrices and Splicing Silencer Matrices were assessed and visualized using the ‘Human Splicing Finder’ website (http://www.umd.be/HSF3/).
[0096] FIG. **2**: Generation of the minigene vectors. The genomic region containing (A) USH2A exon 30 or (B) USH2A exon 31 and flanking sequences were cloned into the pCi-Neo Rho destination vector. This resulted in two minigene vectors which contain the fragments of interest flanked by two rhodopsin exons under the control of a CMV promotor. pDEST_pCi-Neo Rho: pCi-Neo Rho destination vector.
[0097] FIG. **3**: In silico modeling of usherin protein domain architecture after exon 30-31 skipping. (A) Schematic representation of the domain architecture of the large protein isoform of human and zebrafish usherin. The large protein isoforms of human and zebrafish usherin are comprised of the same repetitive protein domain architecture that includes a signal peptide, a laminin G-like domain

(LamG-like), a laminin N-terminal domain (LamNT), 10 EGF-like motifs, four fibronectin type III (FN3) domains, two laminin G domains (LamG), 28 additional FN3 domains, one transmembrane domain and a short intracellular region with a C-terminal class I PDZ binding motif. Skipping of USH2A exons 30-31 results in the exclusion of one FN3 domain. The domain that is lost in human and zebrafish usherin is indicated with a dashed box. Numbers indicate amino acids. (B) 3D homology modeling of USH2A exons 30-31 skipping predicts the removal of exactly one FN3 domain without disturbing the folding of neighboring FN3 domains. The amino acids that are encoded by targeted exons are depicted in dark gray.

[0098] FIG. **4**: Design and characterization of the ush2a.sup.Δexon30-31 zebrafish line. (A) Schematic representation of the exon excision approach. Sanger sequencing confirmed the presence of the anticipated excision in injected embryos (1 day post fertilization (dpf)). Excision of the genomic region containing ush2a exons 30 and 31 resulted in the insertion of two nucleotides (TT) at the repair junction. (B) RT-PCR analysis revealed the absence of ush2a exons 30 and 31 in ush2a.sup.Δexon30-31 larvae (5 days post fertilization (dpf)). Sanger sequencing of the ush2a.sup.Δexon30-31 amplicon confirmed the absence of the targeted exons from the transcript.

[0099] FIG. **5**: Visualization of usherin proteins on retinal sections of wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 zebrafish. (A) Retinal cryosections of wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 larvae (5 days post fertilization (dpf)) stained with antibodies directed against usherin and centrin. Nuclei are counterstained with DAPI. In wild-type larvae, usherin is localized in the periciliary region, in close proximity to centrin. No usherin signal was detected in ush2a.sup.rmc1 zebrafish retinas, whereas in the retinas of wild-type and ush2a.sup.Δexon30-31 larvae usherin was present adjacent to the centrin immunoreactivity. Scale bar: 10 μm. OS: outer segment, CC: connecting cilium, IS: inner segment, ONL: outer nuclear layer. (B) Quantification of anti-usherin signal intensity at the periciliary region. Individual data points represent the mean fluorescence intensity of anti-usherin staining at the periciliary region of all photoreceptors of a single, central section of one larval zebrafish eye. Horizonal bars depict the mean signal intensity within a genotype (n=25-28 eyes). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. **** $P \leq 0.0001$.

[0100] FIG. **6**: Visualization of Adgrv1 proteins on retinal sections of wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 zebrafish. (A) Retinal cryosections of wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 larvae (5 days post fertilization (dpf)) stained with antibodies directed against Adgrv1 and centrin. Nuclei are counterstained with DAPI. A reduced Adgrv1 signal was detected in ush2a.sup.rmc1 zebrafish retinas, whereas in the retinas of wild-type and ush2a.sup.Δexon30-31 larvae usherin was more abundantly present at the photoreceptor periciliary region, in close proximity to centrin. Scale bar: 10 μm. OS: outer segment, CC: connecting cilium, IS: inner segment, ONL: outer nuclear layer. (B) Quantification of anti-Adgrv1 signal intensity at the periciliary region. Individual data points represent the mean fluorescence intensity of anti-Adgrv1 staining at the periciliary region of all photoreceptors of a single, central section of one larval zebrafish eye. Horizonal bars depict the mean signal intensity within a genotype (n=14 eyes). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. **** $P \leq 0.0001$.

[0101] FIG. **7**: Immunohistochemistry on retinal sections of wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 zebrafish. (A) Retinal cryosections of 6 days post fertilization (dpf) were analyzed for rhodopsin localization. Nuclei were counterstained with DAPI. In the retinas of wild-type and ush2a.sup.Δexon30-31 larvae rhodopsin was predominantly present in the photoreceptor outer segments, whereas in ush2a.sup.rmc1 zebrafish retinas rhodopsin signal was also detected in the outer nuclear layer, and more specifically the inner segments as indicated by arrowheads. Scale bar: 10 μm. ROS: rod outer segment, COS: cone outer segment, ONL: outer nuclear layer, INL: inner nuclear layer. (B) Scatterplot of rhodopsin localization spots in the outer nuclear layer of 6 dpf zebrafish obtained by manual counting and plotted per counted eye (n=12-14). Horizonal bars

depict the mean signal intensity within a genotype. The amount of rhodopsin localization spots in the outer nuclear layer is significantly lower in wild-type and ush2a.sup.Δexon30-31 retinas as compared to ush2a.sup.rmc1 retinas. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. * P≤0.05.

[0102] FIG. **8**: Identification of potent ASOs using a minigene splice assay. HEK293T cells were co-transfected with the minigene containing USH2A exon 31 and either ASO_31_1 or ASO_31_2 in a 250 nM concentration. The upper amplicon represents the transcript containing USH2A exon 31, whereas the lower amplicon represents the transcript lacking the targeted exon. GAPDH amplification is shown as a loading control. ASO: antisense oligonucleotide; PCR(−): negative PCR control.

[0103] FIG. **9**: Validation of dual ASO-induced skipping of USH2A exon 30 using a minigene splice assay. HEK293T cells were co-transfected with the minigene vector and either ASO_30_1, ASO_30_2 or a cocktail of ASO_30_1 and ASO_30_2 in a final concentration of 100 or 250 nM. The upper amplicon represents the transcript containing USH2A exon 30, whereas the lower amplicon represents the transcript lacking USH2A exon 30. ASO: antisense oligonucleotide; PCR(−): negative PCR control.

[0104] FIG. **10**. ASOs induce a dose-dependent skipping of USH2A exon 30 and 31. HEK293T cells are co-transfected with (A) the USH2A exon 30 minigene vector and different concentrations of ASOs targeting USH2A exon 30 or (B) the USH2A exon 31 minigene vector and different concentrations of ASO targeting USH2A exon 31. Each transfection resulted in an increase in exon skipped transcripts with increasing concentrations of ASO. Non-skipped amplicons and amplicons in which the targeted USH2A exon is skipped are indicated adjacent to the gel image. GAPDH amplification is shown as a loading control. ASO: antisense oligonucleotide; mmASO: mismatch ASO; PCR(−): negative PCR control.

[0105] FIG. **11**: Validation of ASO-induced dual exon skipping for USH2A exons 30-31 in WERI-Rb-1 cells. Co-transfection of WERI-Rb-1 cells with ASOs targeting USH2A exons 30 and 31 resulted in combined skipping of the exons of interest. Co-transfection of WERI-Rb-1 cells with ASOs targeting USH2A exons 30 and 31 also resulted in additional skipping of exon 32. GAPDH amplification is shown as a loading control. ASO: antisense oligonucleotide; mmASO: mismatch ASO; PCR(−): negative PCR control.

DESCRIPTION OF THE SEQUENCES

TABLE-US-00001 TABLE 1 Sequences SEQ ID NO: Name 1 USH2A genomic DNA wild-type 2 USH2A exon 30-31 and flanking genomic sequence 3 USH2A exon 30 sequence 4 USH2A exon 31 sequence 5 ASO_30_1 target site and flanking sequences (+10 nt) 6 ASO_30_2 target site and flanking sequences (+10 nt) 7 ASO_31_1 target site and flanking sequences (+10 nt) 8 ASO_31_2 target site and flanking sequences (+10 nt) 9 ASO_30_1 target site and flanking sequences (+5 nt) 10 ASO_30_2 target site and flanking sequences (+5 nt) 11 ASO_31_1 target site and flanking sequences (+5 nt) 12 ASO_31_2 target site and flanking sequences (+5 nt) 13 ASO_30_1 14 ASO_30_2 15 ASO_31_1 16 ASO_31_2 17 T7 promoter sequence 18 Region complementary to constant oligo 19 constant oligo 20 target-specific oligo 5′ ush2a Δexo30-31 21 target-specific oligo 3′ ush2a Δexo30-31 22 ush2a wild-type forward primer 23 ush2a wild-type reverse primer 24 ush2a Δexon30-31 forward primer 25 ush2a Δexon30-31 reverse primer 26 ush2a exon 28-33 forward primer 27 ush2a exon 28-33 reverse primer 28 USH2A intron 29-30 forward primer 29 USH2A intron 29-30 reverse primer 30 USH2A intron 30-31 forward primer 31 USH2A intron 30-31 reverse primer 32 Rho exon 3-5 forward primer 33 Rho exon 3-5 reverse primer 34 ush2a exon 28-33 forward primer 35 ush2a exon 28-33 reverse primer 36 GAPDH forward primer 37 GAPDH reverse primer 38 mmASO_30_1 39 mmASO_30_2 40 mmASO_31_2

EXAMPLES

[0106] In this study, we explored dual exon skipping as a future treatment option for patients with RP caused by mutations in the USH2A gene. Based on in silico protein analysis, and the reported

presence of multiple RP-associated protein-truncating mutations, we opted to target exons 30-31 of human USH2A. Calculations based on available carrier frequencies in the general population for the various reported pathogenic variants Mutations in USH2A exons 30-31 (gnomAD v2.1.1) are estimaindicate that ˜ted to affect 22,000 individuals worldwide suffer from USH2A-associated disease caused by (a) variant(s) in these exons. and skipping of this combination of exons is predicted to result in a transcript encoding a shortened usherin protein that lacks exactly one of the 10 repetitive fibronectin type III (FN3) domains. To model the functional consequences of our exon skipping approach, we employed CRISPR/Cas9 and excised the orthologous target exons from the zebrafish genome. This resulted in the ush2a.sup.Δexon30-31 zebrafish line in which usherin protein expression was restored in photoreceptors, and both the localization of rhodopsin to the photoreceptor outer segment and the formation of the USH2 protein complex at the periciliary membrane was rescued. To translate these findings into a future treatment in man, we identified and in vitro validated ASOs with a high, sequence-specific dual exon skipping potential. Together, our data suggest that a carefully designed dual exon skipping approach, in which two exons remove a single protein domain, could be highly promising as a future treatment option for USH2A-associated RP.

Materials and Methods

Zebrafish Ethics, Maintenance and Husbandry

[0107] Animal experiments were conducted in accordance with the Dutch guidelines for the care and use of laboratory animals (Wet op de Dierproeven 1996) and European regulations (Directive 86/609/EEC), as approved by the Dutch Ethics committee of the Central Committee Animal Experimentation (Centrale Commissie Dierproeven [CCD]; Protocol #RU-DEC2016-0091). Wild-type Tupfel Longfin (TL) zebrafish and the previously described ush2a.sup.rmc1 mutants (c.2337_2342delinsAC; p.Cys780GInfsTer32) were used. Zebrafish were maintained and raised according to standard methods. Both adult and larval zebrafish were kept at a light-dark regime of 14 hours of light and 10 hours of darkness. Adult zebrafish were daily fed twice with Gemma Micro 300 dry pellets (#13177, Zebcare, Nederweert, The Netherlands) at ˜5% body weight and once with artemia. Embryos were obtained from natural spawning.

Multiple Sequence Alignment

[0108] A multiple sequence alignment of the human usherin protein (ENSP00000305941_3) and zebrafish usherin protein (ENSDARP00000080636_3) was generated using AlignX in the Vector NTI software package (Vector NTI Advance 11).

In Silico Modeling of the Effect of USH2A Exons 30-31 Skipping on FN3 Protein Domain Structure

[0109] The 5202 amino acid usherin protein sequence was selected from the UniProtKB database (www.uniprot.org/; acc. #075445) and was used as template protein sequence for modeling the effect of USH2A exons 30-31 skipping on the 3D protein domain structure of usherin. The effect of USH2A exons 30-31 skipping on the usherin protein structure was modeled in a chain of one laminin G (Lam G) domain followed by three consecutive fibronectin type III (FN3) domains. For prediction of the wild-type protein structure LamG (2), FN3_5, FN3_6 and FN3_7 were included, of which FN3_6 is encoded by USH2A exons 30-31. The structural model of usherin.sup.Δexon30-31 included LamG_2, FN3_5, FN3_7 and FN3_8. The AlphaFold2 (previously described) modeling script was used to generate the structural models of both wild-type and usherin.sup.Δexon30-31 proteins by employing standard parameters.

CRISPR/Cas9 Genome-Editing Design

[0110] Target sites for single guide RNAs (sgRNAs) to cleave in introns 29 and 31 of zebrafish ush2a (NCBI accession XM_009293147.3) were identified with the online web tool CHOPCHOP. sgRNAs for which no off-target sites were predicted and which had the highest predicted efficiency score were selected for synthesis. Synthesis of sgRNAs was performed as described previously. In brief, templates for in vitro sgRNA transcription were generated by annealing a constant

oligonucleotide encoding the reverse complement of the tracrRNA tail to a target-specific oligonucleotides containing the T7 promoter sequence (5′-TAATACGACTCACTATA-3′-SEQ ID NO: 17), the 20-base target sequence, and a region (5′-GTTTTAGAGCTAGAAATAGCAAG-3′-SEQ ID NO: 18) complementary to the constant oligonucleotide. Phusion™ High-Fidelity DNA Polymerase (#M0530L, New England Biolabs, Ipswich, MA, USA) was used to fill the ssDNA overhang after which the template was purified using the GenElute™ PCR clean-up kit (#NA1020-1KT, Sigma-Aldrich, St. Louis, MO, USA). The template was used for the in vitro transcription of the sgRNAs using the T7 MEGAshortscript™ Kit (#AM1354, Thermo Fisher Scientific, Waltham, MA, USA). Obtained transcripts were purified using the MEGAclear™ Transcription Clean-Up Kit (#AM1908, Thermo Fisher Scientific, Waltham, MA, USA). Oligonucleotides used for sgRNA synthesis are listed in Table 2.

TABLE-US-00002 TABLE 2 Oligonucleotide sequences used for sgRNA synthesis. Oligo Name Sequence (5′ > 3′) SEQ ID NO constant oligo AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAAC 19 GGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAA C target-specific oligo CCGCTAGC**TAATACGACTCACTATA***GGGTGAGAATCCA* 20 5′ ush2a.sup.Δexo30-31 *ATTGGGA*GTTTTAGAGCTAGAAATAGCAAG target-specific oligo CCGCTAGC**TAATACGACTCACTATA***GGGCTAAAAAGAA* 21 3′ ush2a.sup.Δexo30-31 *TTGATTG*GTTTTAGAGCTAGAAATAGCAAG T7 promoter sequence in bold. Gene specific region in italics. Overlapping regions of the constant and target-specific oligonucleotides are underlined.

Microinjections

[0111] For the generation of the ush2a.sup.Δexon30-31 zebrafish (deposited as ush2a.sup.rmc21 in ZFIN), the 5′ sgRNA, 3′ sgRNA and commercial Alt-R® S.p. Cas9 Nuclease V3 (#1081059, IDT, Newark, NJ, USA) were co-injected. To avoid preferential in vivo binding of Cas9 to either sgRNA, individual sgRNA-Cas9 complexes were prepared and mixed together prior to injection. For this, the individual mixtures were incubated at 37° C. for 5 minutes after which they were combined. The final injection mix contained 80 ng/μl 3′ sgRNA, 80 ng/μl 5′ sgRNA, 800 ng/μl Cas9 protein, 0.2 M KCl and 0.05% phenol red. Injection needles (#TW120F-3, World Precision Instruments, Friedberg, Germany) were prepared using a micropipette puller (Model P-97, Sutter Instrument Company, Novato, CA, USA). Wild-type zebrafish embryos were collected after natural spawning and injected at the single cell stage with 1 nl of injection mixture using a Pneumatic PicoPump (#SYS-PV820, World Precision Instruments, Friedberg, Germany). After injection, embryos were raised at 28.5° C. in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl.sub.2, and 0.33 mM MgSO.sub.4) supplemented with 0.1% (v/v) methylene blue. At 1 day post fertilization (dpf), part of the injected embryos was analyzed for the presence of the anticipated exon deletion using genomic PCR analysis. The remainder of the injected embryos were raised to adulthood.

Genotyping

[0112] Genomic DNA was extracted from whole larvae (1 dpf) or caudal fin tissue from adult zebrafish. Tissue was lysed in 25 μl (larvae) or 75 μl (fin tissue) lysis buffer (40 mM NaOH, 0.2 mM EDTA) at 95° C. for 20 minutes. The lysed samples were neutralized with 10% 1M TRIS-HCl (pH 7.5) and diluted 10 times with milli-Q water. 1 μl of diluted sample was used as a template in PCR reactions to amplify the zebrafish ush2a.sup.Δexon30-31 allele and the corresponding wild-type zebrafish ush2a allele. For PCR analysis, the Q5 High-Fidelity DNA Polymerase kit (#M0491L, New England Biolabs, Ipswich, MA, USA) was employed. All primer sequences are listed in Table 1 (SEQ ID NO 22-37). The presence or absence of the ush2a.sup.Δexon30-31 allele was confirmed by Sanger sequencing.

Immunohistochemistry, Histology and Quantification of Fluorescent Signal Intensity

[0113] Zebrafish ush2a.sup.Δexon30-31, ush2a.sup.rmc1 and strain-matched wild-type larvae (5

dpf) were cryoprotected with 10% sucrose in PBS for 10 minutes prior to embedding in OCT compound (Tissue-Tek, #4583, Sakura, Alphen aan den Rijn, The Netherlands). After embedding, samples were snap frozen in liquid nitrogen-cooled isopentane and sectioned following standard protocols. Cryosections (7 μm thickness along the lens/optic nerve axis) were rinsed with PBS, permeabilized for 20 minutes with 0.01% Tween-20 in PBS and blocked for 1 hour with blocking buffer (10% normal goat serum and 2% bovine serum albumin in PBS). Antibodies diluted in blocking buffer were incubated overnight at 4° C. Secondary antibodies were also diluted in blocking buffer and incubated together with DAPI (1:8000; D1306; Molecular Probes, Eugene, OR, USA) for 1 hour. Sections were post fixed with 4% paraformaldehyde for 10 minutes and mounted with Prolong Gold Anti-fade (P36930; Molecular Probes, Eugene, OR, USA). The following primary antibodies and dilutions were used: rabbit anti-usherin (1:500; #27640002, Novus Biologicals, Centennial, CO, USA), mouse anti-centrin (1:500; #04-1624, Millipore, Burlington, MA, USA) and rabbit anti-Adgrv1 (1:100; #DZ41033, Boster Bio, Pleasanton, CA, USA). Secondary antibodies (Alexa Fluor 568 goat anti-rabbit (#A11011, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 647 goat anti-mouse (#A21237, Thermo Fisher Scientific, Waltham, MA, USA)) were used in a 1:800 dilution. Images were taken using a Zeiss Axio Imager fluorescence microscope equipped with an AxioCam MRC5 camera (Zeiss, Jena, Germany). Quantification of the fluorescent signal intensity of anti-usherin and anti-Adgrv1 immunoreactivity was performed using Fiji version (v.) 1.47 software as described previously. Upon identification of the areas of the connecting cilia, the maximum and minimum gray value of usherin or Adgrv1 immunofluorescence in those areas was measured. The difference between those values was calculated for each individual photoreceptor cell after which the mean difference per retina was plotted. All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set at $p<0.05$.

[0114] To assess rhodopsin localization in the larval retina, larvae (6 dpf) from homozygous ush2a.sup.Δexon30-31 ush2a.sup.rmc1 and strain-matched wild-type controls were sampled 100 minutes post light onset. Larvae were fixed in darkness overnight at 4° C. using 4% paraformaldehyde, dehydrated using methanol series with an ascending concentration, transferred to 100% methanol for an overnight incubation followed by storage at −20° C. Upon embedding, larvae were rehydrated in descending methanol series to 0.1% PBS-Tween-20. Afterwards, larvae were cryoprotected with 10% sucrose in 0.1% PBS-Tween-20 for 15 minutes, followed by an incubation in 30% sucrose in 0.1% PBS-Tween-20 for 1 hour at room temperature. Larvae were then embedded, snap frozen and sectioned as described above. Cryosections were rinsed with PBS, permeabilized for 2 minutes with 0.1% Tween-20 in PBS and, immersed in 10 mM Sodium Citrate at pH 8.5 and heated for 1 min at 121° C. in the autoclave. Cryosections were subsequently washed in 0.1% Tween-20 in PBS and blocked for 1 hour with blocking buffer (10% non-fat dry milk and 0.1% Tween-20 in PBS). Primary antibody (mouse anti-rhodopsin, 1:4000, #NBP2-59690, Novus Biologicals, Centennial, CO, USA) diluted in blocking buffer was incubated overnight at 4° C. Secondary antibody (Alexa Fluor 488 goat anti-mouse, 1:800, #A11029, Thermo Fisher Scientific, Waltham, MA, USA) was also diluted in blocking buffer and incubated together with DAPI (1:8000; #D1306; Thermo Fisher, Waltham, MA, USA) for 1.5 hour. Sections were mounted and images were taken as described above. Rhodopsin levels were quantified by manual counting. For this, all pictures were taken using the same settings after which the mislocalisation spots in the region of interest (outer nuclear layer), blinded and analyzed independently by two individuals. For all pictures mean counts were calculated and analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set at $p<0.05$.

Antisense Oligonucleotides

[0115] The sequence of USH2A exon 30 and 31 and the 50 bp upstream and downstream flanking intronic sequences were analyzed to identify potential ASO target sites as described previously (Slijkerman, R, Exon Skipping and Inclusion Therapies. 2018, Springer. p. 519-530). (FIG. **1**).

Briefly, the presence of exonic splice enhancer motifs was assessed using the 'Human Splicing Finder' website (www.umd.be/HSF3/; Date accessed: Dec. 16, 2020) and RNA structure and free energy predictions were performed using freely available database tools (www.unafold.org/; rna.urmc.rochester.edu/RNAstructureWeb/index.html). ASOs were designed to have a Tm≥48° C., a GC content between 40-60% and a length of 17-23 nt. Subsequently, for each targeted exon, the 2-5 most optimal ASOs were purchased from Eurogentec (Liège, Belgium) containing 2′-O-(2-methoxyethyl) modified ribose groups and a fully phosphorothioated backbone. The matching control ASOs all contain four mismatches relative to the target sequence. All ASOs were dissolved in phosphate-buffered saline (PBS) before use. ASO sequences are listed in Table 3.

TABLE-US-00003 TABLE 3 Antisense oligonucleotide sequences and characteristics.

RNA oligonucleotide	Sequence (5′ > 3′)	Length (nt)	GC content (%)	SEQ ID NO
ASO_30_1	CACUUUGUGGAGCUGUGAAGG	21	52	13
ASO_30_2	GCUGUAUCCAUUUAAGCUGCG	19	53	14
ASO_31_1	GUAGCAAGCCUGUCAAUAUGCC	22	50	15
ASO_31_2	CUUCUUGUGGAGUAGAGAUGUU	22	41	16
mmASO_30_1	CACUUUGUAUAACUGUUAAGG	21	33	38
mmASO_30_2	GCUAUAUCCAUUUAAAAUACG	19	47	39
mmASO_31_2	CUUAUUGUGUAUUAAAGAUGUU	22	22	40

ASO: antisense oligonucleotide; mm: mismatch; nt: nucleotide; Mismatches with the target sequence are underlined. All ASO were ordered with 2′-O-(2-methoxyethyl) modified ribose groups and a fully phosphorothioated backbone.

Minigene Vectors

[0116] The genomic region containing human USH2A exon 30 or USH2A exon 31, together with 300-1000 bp of flanking up- and downstream intronic sequence, was cloned into a pDONR201 vector using Gateway cloning technology. Primer sequences are listed in Table 2. The complete insert of the donor vector was sequence verified and subsequently cloned into the pCI-Neo-Rho destination vector, which enables the expression of the fragment of interest flanked by two rhodopsin exons. This resulted in 2 minigene vectors, either containing human USH2A exon 30 or USH2A exon 31 (FIG. **2**).

Cell Culture

[0117] HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (#D0819, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (#F7524, Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin (#P4333, Sigma-Aldrich, St. Louis, MO, USA) and 1% sodium pyruvate (#S8636, Sigma-Aldrich, St. Louis, MO, USA). Cells were passaged twice per week upon standard trypsinization (#DF0152-15-9, Thermo Fisher Scientific, Waltham, MA, USA). WERI-Rb-1 cells were cultured in RPMI-1640 (#22409-015, Gibco Waltham, MA, USA) supplemented with 15% (v/v) fetal bovine serum ((#F7524, Sigma-Aldrich, St. Louis, MO, USA), 2% HEPES (#H0887, Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin ((#P4333, Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in suspension and maintained by addition of fresh medium or replacement of medium every 3 to 4 days.

Transfection of ASOs and Minigene Vectors in HEK293T Cells

[0118] HEK293T cells were seeded at a concentration of ˜0.2×10.sup.5 cells per well in a 24-well plate and grown for 24 hours at 37° C. in a total volume of 0.5 ml medium. Cells were (co-)transfected with 500 ng of the minigene vector and the indicated amount of ASO, calculated as the final concentration in the culture medium after ASO delivery. The transfection mixture furthermore contained 3 μl Fugene® HD Transfection Reagent (#E2311, Promega, Madison, WI, USA), and was prepared in a final volume of 50 μl Opti-Mem (#31985-047, Gibco Waltham, MA, USA), according to manufacturer's protocol. Two wells per condition were treated. After incubation for 24 hours at 37° C., cells were washed once with PBS and harvested for RNA isolation.

Transfection of ASOs in WERI-Rb-1 Cells

[0119] Transfections were performed on adherend cells. For this purpose, all wells of a 12-well

plate were coated with Poly-L-Lysine (#P4707, Sigma Aldrich, Saint Louis, MO, USA) by adding 0.5 ml Poly-L-Lysine to each well. After a 90-minute incubation at 37° C., Poly-L-Lysine was removed from the wells and wells were washed three times with PBS and air-dried for 30 minutes. Next, WERI-Rb-1 were seeded at a concentration of 1.0×10.sup.6 cells per well in a 12-well plate and incubated for 48 hours at 37° C. Cells were subsequently transfected with the ASO of interest using Lipofectamine™ 2000 transfection reagent (#11668019, Thermo Fisher Scientific, Waltham, MA, USA) at a 2:1 (volume:weight) ratio between Lipofectamine™ 2000 and ASO. First, individual Lipofectamine™ 2000/Opti-MEM (50 μl) mixtures and an ASO/Opti-MEM (50 μl) mixtures were prepared. Both mixtures were individually incubated at room temperature for 5 minutes. Next, the ASO and Lipofectamine™ mixtures were mixed together and incubated at room temperature for an additional 10 minutes before being added to the cells. After a 24 hour incubation at 37° C., cells were washed once with PBS and harvested for RNA isolation.

RNA Isolation and cDNA Synthesis

[0120] Total RNA was isolated from transfected HEK293T cells or WERI-Rb-1 cells using the Nucleospin RNA II isolation kit (#740955.250, MACHEREY-NAGEL, Düren, Germany), according to manufacturer's protocol whereas total RNA from zebrafish larvae (5 dpf) was extracted using the RNeasy® Micro kit (#74004, Qiagen, Hilden, Germany). For cDNA synthesis from HEK293T RNA, the iScript™ cDNA synthesis kit (#1708891, Bio-Rad, Hercules, CA, USA) was used with 0.5 μg total RNA as input. From WERI-Rb-1 and zebrafish RNA, cDNA was synthesized using SuperScript™ IV Reverse Transcriptase (#18090010, Thermo Fisher Scientific, Waltham, MA, USA), Oligo(dT).sub.12-18 primer (#18418012, Thermo Fisher Scientific, Waltham, MA, USA) and 0.1-0.3 μg total RNA, according to manufacturer's protocol.

Transcript Analysis

[0121] For the exon skipping experiments using the minigene splice vectors, the target region was amplified from the synthesized cDNA using Taq polymerase (New England Biolabs, M0491L, Ipswich, MA) and a forward primer and reverse primer located in exons 3 and 5 of the human RHO gene, respectively. For the experiments in WERI-Rb-1 cells and zebrafish larvae, the target region was amplified from the synthesized human or zebrafish cDNA using Q5@ High-Fidelity DNA Polymerase (#M0491L, New England Biolabs, Ipswich, MA, USA). For the exon skipping experiments in HEK293T cells and WERI-Rb-1 cells, primers amplifying GAPDH using Taq polymerase (New England Biolabs, M0491L, Ipswich, MA) were employed as a control. All primer sequences are listed in Table 1 (SEQ ID NO: 22-37). Amplified fragments were separated on a 1% agarose gel and sequence-verified by Sanger sequencing.

Results

USH2A Exons 30-31 is a Promising Target for Exon Skipping

[0122] USH2A exons 30 and 31 (306 nucleotides) encode exactly one fibronectin type III (FN3) domain. In addition, numerous unique loss-of-function mutations have been reported in those exons, making important for mutation them targets exon skipping (USH2A LOVD database, databases.lovd.nl/shared/variants/USH2A/unique). Skipping this combination of exons will maintain the open reading frame of the USH2A transcript, and is predicted to result in the production of a slightly shortened usherin protein lacking exactly one FN3 domain (FIG. **3**A). 3D homology modeling predicted human usherin.sup.Δexon30-31 and usherin.sup.Δexon39-40 to show a nearly identical protein domain structure in which folding of neighboring FN3 domains is not disturbed (FIG. **3**B).

Generation of the ush2a.sup.Δexon30-31 Zebrafish Line Using CRISPR/Cas9 Technology

[0123] We and others have previously established the translational value of zebrafish models to study the retinal phenotype of Usher syndrome (Dona, M., et al. 2018. 173: p. 148-159, Dulla, K., et al, Molecular Therapy, 2021; Noel, N. C., I. M. MacDonald, and W. T. Allison, Biomolecules, 2021. 11 (1): p. 78). The human and zebrafish usherin protein share a similar protein domain architecture and an overall sequence identity of 52% (Dona et al, supra). The protein region

encoded by zebrafish and human USH2A exons 30-31 shows a 61% sequence identity between human and zebrafish. Similar to the human situation, the in-frame deletion of zebrafish ush2a exons 30-31 is predicted to result in a shortened protein (usherin.sup.Δexon30-31) from which exactly one FN3 domain is lost (FIG. **1**A).

[0124] To assess the effects of dual exon skipping therapy on usherin protein function, we adopted CRISPR/Cas9 technology to generate a stable zebrafish line from which the genomic region encompassing ush2a exons 30-31 was specifically excised. For this, Cas9 protein and two sgRNAs, one targeting the genomic region upstream and one targeting the genomic region downstream of the exonic targets, were injected in fertilized embryos (FIG. **4**A). Correct exon excision was confirmed by genomic PCR and Sanger sequencing. A stable homozygous zebrafish ush2a exons 30-31 excision lines was bred from a germline-positive founder fish, and designated ush2a.sup.Δexon30-31. Homozygous ush2a.sup.Δexon30-31 fish were viable and did not display any abnormalities in overall body morphology, development, or swimming behavior.

[0125] To determine the effect of excision on transcriptional level, total RNA was isolated from pairs of homozygous ush2a.sup.Δexon30-31 larvae. RT-PCR analysis using a forward and reverse primer in respectively exons 28 and 33 of the zebrafish ush2a gene detected a shortened PCR fragment in the ush2a.sup.Δexon30-31 zebrafish in the absence of any clear alternatively spliced ush2a transcripts (FIG. **4**B). Sanger sequencing confirmed the expression of the expected ush2a transcript exclusively lacking the anticipated target exons from the ush2a transcripts derived from the ush2a.sup.Δexon30-31 larvae.

Excision of ush2a Exons 30-31 Restores Usherin Protein Expression in Genetically Modified Zebrafish

[0126] To investigate whether the skipping of ush2a exons 30-31 resulted in the translation and correct localization of the usherin.sup.Δexon30-31 protein in photoreceptor cells, an immunohistochemical analysis of usherin expression was performed. In the wild-type zebrafish retina, usherin is expressed at the periciliary region of zebrafish photoreceptors (Dona, M., et supra; Dulla, K., supra). Antibodies directed against the intracellular region of the usherin protein and antibodies directed against the connecting cilium marker centrin were used to co-stain unfixed retinal cryosections of 5 dpf wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 zebrafish larvae (FIG. **5**A). Anti-usherin immunoreactivity is absent in photoreceptors of ush2a.sup.rmc1 larvae. As hypothesized, the usherin.sup.Δexon30-31 protein localizes at the photoreceptor periciliary region, adjacent to the connecting cilium marker centrin, similar to full length usherin localization in strain- and age-matched wild-type larvae. The mean intensity of the anti-usherin fluorescence signals at all periciliary regions in the middle section of each eye, was quantified using an automated Fiji script. This analysis corroborated that skipping of ush2a exons 30-31 leads to a restoration in usherin protein levels (FIG. **5**B; means, standard deviations and n-values are shown in Table 4). As also previously published, usherin immunoreactivity is significantly decreased in ush2a.sup.rmc1 mutants as compared to wild-type controls (Dulla, K., et al. supra). Skipping of exons 30-31 resulted in a restoration of normal anti-usherin fluorescent signal intensities.

TABLE-US-00004 TABLE 4 Means, standard deviations and n-values of anti- usherin fluorescence signal quantification. Zebrafish line Mean Standard deviation n wild-type 42.05 10.90 28 ush2a.sup.rmc1 16.15 8.05 25 ush2a.sup.Δexon30-31 45.77 11.50 28 n: number of zebrafish.

Skipping of ush2a Exons 30-31 does not Alter Adgrv1 Localization in Zebrafish Photoreceptors

[0127] The correct localization of the usherin.sup.Δexon30-31 protein indicates that the FN3 domain encoded by exons 30-31 can be missed without interfering with usherin expression and subcellular localization in zebrafish photoreceptors. As a next step, the influence of the excision of the target exons on the expression and localization of usherin interaction partners was analyzed. In humans, usherin and the other known USH2 proteins, whirlin and ADGRV1, form a dynamic protein complex (Yang, J., et al., PLOS genetics, 2010. 6 (5): p. e1000955; van Wijk, E., et al., Human molecular genetics, 2006. 15 (5): p. 751-765). We previously showed that defects of

usherin led to a reduction in localization of the USH2 complex members, whirlin and Adgrv1, at the photoreceptor periciliary membrane of ush2a.sup.rmc1 zebrafish larvae. To analyze whether the excision of the target exons resulted in restoration of Adgrv1 expression in zebrafish photoreceptors, antibodies directed against the N-terminal region of Adgrv1 and antibodies directed against the connecting cilium marker centrin were used to co-stain unfixed retinal cryosections of 5 dpf wild-type, ush2a.sup.rmc1 and ush2a.sup.Δexon30-31 zebrafish larvae (FIG. **6**A). Anti-Adgrv1 immunoreactivity is indeed absent in photoreceptors of ush2a.sup.rmc1 larvae and, as hypothesized, the usherin.sup.Δexon30-31 protein is able to restore Adgrv1 protein expression at the photoreceptor periciliary region. The mean intensity of the anti-Adgrv1 fluorescence signals at all periciliary regions in the middle section of each eye, was quantified using an automated Fiji script. As previously published, Adgrv1 immunoreactivity is significantly decreased in photoreceptors of ush2a.sup.rmc1 mutants as compared to wild-type controls (FIG. **6**B) (Dona, M., et al., 2018. 173: p. 148-159). Skipping of exons 30 and 31 resulted in a restoration of normal anti-Adgrv1 fluorescent signal intensities. This suggests that, similar to native usherin, the usherin.sup.Δexon30-31 protein is able to scaffold the USH2 protein network in the periciliary region.

Skipping of ush2a Exons 30-31 Restores Rhodopsin Trafficking in Genetically Modified Zebrafish

[0128] Loss of usherin function was previously shown to lead to defective rhodopsin transport from the inner segment to the outer segment of photoreceptors (Toms, M., et al., Human molecular genetics, 2020. 29 (11): p. 1882-1899). We therefore investigated if skipping of ush2a exons 30-31 is able to restore rhodopsin trafficking. In the retinas of 6dpf wild-type larvae, rhodopsin is present in the outer segments (FIG. **7**A). In line with Toms et al supra, cells in which rhodopsin was partially localized to the inner segments of photoreceptor cells were occasionally observed in wild-types. In retinas of ush2a.sup.rmc1 larvae, the amount of cells per retinal section was significantly increased ($P<0.05$; one way ANOVA followed by Tukey's multiple comparison test). Quantification of number of cells with rhodopsin immunoreactivity in the inner segments revealed a significant reduction of rhodopsin localization in the outer nuclear layer of the ush2a.sup.Δexon30-31 larvae as compared to ush2a.sup.rmc1 larvae ($P<0.05$; one way ANOVA followed by Tukey's multiple comparison test), while those levels were comparable to levels in retinas of wild-type larvae (FIG. **7**B; means, standard deviations and n-values are shown in Table 5). This suggests that the usherin.sup.Δexon30-31 protein is able to support normal ciliary trafficking of rhodopsin to the photoreceptor outer segment.

TABLE-US-00005 TABLE 5 Means, standard deviations and n-values for the quantification of rhodopsin localization in the outer nuclear layer. Zebrafish line Mean Standard deviation n wild-type 7.82 8.54 14 ush2a.sup.rmc1 16.25 8.96 12 ush2a.sup.Δexon30-31 7.04 6.88 13

Identification of Splice Modulating Antisense Oligonucleotides

[0129] Based on the ability of usherin.sup.Δexon30-31 to properly localize in photoreceptors and restore USH2 protein complex formation and rhodopsin trafficking, we aimed to design and in vitro validate ASOs that specifically induce combined skipping of exon 30 and 31 from USH2A pre-mRNA. For each exon of interest, 2-5 ASOs were designed to target either the intron-exon boundaries, or exonic splicing enhancer (ESE) motifs within the exons. With the use of in silico analysis, parameters for (lack of) secondary structure formation, thermodynamic properties, and sequence selectivity were taken into account to minimize potential off-target effects. As a non-binding control, mismatch ASOs (mmASO) were used that contained four mismatches relative to the target sequence. All ASOs contain 2′-O-(2-methoxyethyl) modified ribose groups and a fully phosphorothioated backbone. To swiftly identify the most potent ASO for each of the target exons, the designed ASOs were co-transfected with the minigene vector in HEK293T cells at a 250 nM concentration, and screened for their potential to induce exon skipping by RT-PCR (data not shown). Because the genomic region spanning USH2A exon 30 and USH2A exon 31 (˜22 kb) exceeds the practical limitations of Gateway cloning technology, individual vectors with either

USH2A exon 30 and flanking sequence, or USH2A exon 31 and flanking sequence, were used in these experiments. For USH2A exon 31, we identified two ASO that showed high exon skipping potential in the minigene splice assays (FIG. **8**). Simultaneous transfection of two ASOs directed against different parts of USH2A exon 30 showed even more satisfactory exon skipping (FIG. **9**). ASOs with highest exon skipping potential as proven in the minigene splice assays, and their accompanied non-binding controls, are listed in Table 3.

Antisense Oligonucleotides Induce a Concentration-Dependent Increase of Exon Skipping in Minigene Splice Assays

[0130] After identifying ASOs with high exon skipping potential in the initial ASO potency screen, we lowered ASO concentrations in order to provide pharmacodynamic proof of concept for ASO-induced exon skipping. As shown in FIG. **10**, for both ASOs (or in case of exon 30, combination of ASOs), we observed a dose-dependent increase in exon skipped transcripts with increasing concentrations of ASO. To exclude non-sequence specific ASO-induced effects or transfection reagents-induced effects on splicing, we co-transfected the minigene vector with the mmASO. In none of the cases, the mmASO was able to induce splice modulation. All exon-exon boundaries of the amplicons were sequence verified.

Antisense Oligonucleotides Induce Dual Exon Skipping in WERI-RB1 Cells

[0131] The retinoblastoma-derived WERI-Rb-1 cell line (McFall, R. C., T. W. Sery, and M. Makadon, Cancer research, 1977. 37 (4): p. 1003-10) was obtained in order to evaluate dual exon skipping potential of the identified ASOs on endogenously expressed USH2A. USH2A transcripts were analyzed by RT-PCR using primers in exon 28 and exon 33. Co-transfection of WERI-Rb-1 cells with selected ASOs targeting exon 30 and exon 31 in a 1:1 ratio for each therapeutic target (25 nM ASO_30A, 25 nM ASO_30E and 50 nM ASO_31B) resulted in the anticipated dual exon skipping effect (FIG. **11**). Co-transfection of WERI-Rb-1 cells with these ASOs also resulted in a faint smaller splice product, which has been verified by sequencing as transcript in which not only the target exons are skipped, but also exon 32 (data not shown).

Claims

1. A set of antisense oligonucleotides for skipping of exon 30 and 31 that bind to and/or are complementary to a polynucleotide with the nucleotide sequence as shown in SEQ ID NO: 1.

2. The set of antisense oligonucleotides for skipping of exon 30 and 31 according to claim 1, wherein each antisense oligonucleotide has a length of from about 8 to about 40 nucleotides.

3. The set of antisense oligonucleotides for skipping of exon 30 and 31 according to claim 1, wherein the set comprises at least two antisense oligonucleotides that comprise or consist of SEQ ID NO: 13, 14, 15 or 16.

4. The set of antisense oligonucleotides for skipping of exon 30 and 31 according claim 1, wherein each antisense oligonucleotide comprises an 2′-O alkyl phosphorothioate, such as 2′-O-methyl modified ribose (RNA), 2′-O-ethyl modified ribose, 2′-O-methoxyethyl modified ribose, 2′-O-propyl modified ribose, and/or substituted derivatives of these modifications such as halogenated derivatives.

5. The set of antisense oligonucleotides for skipping of exon 30 and 31 as defined in claim **6**, wherein each antisense oligonucleotide comprises a 2′-O-methoxyethyl modified ribose and a phosphorothioate backbone.

6. (canceled)

7. A pharmaceutical composition comprising the set of antisense oligonucleotides for skipping exons 30 and 31 according to claim 1 and a pharmaceutically acceptable excipient.

8. A pharmaceutical composition according to claim 7, wherein the pharmaceutical composition is for intravitreal administration or intratympanic administration.

9. A pharmaceutical composition according to claim 7, wherein the pharmaceutical composition is

for intravitreal administration and is dosed in an amount ranged from 0.1 and 15 mg of total antisense oligonucleotides for redirecting splicing per eye or per ear.

10. (canceled)

11. (canceled)

12. (canceled)

13. A method for the treatment of a USH2A related disease or condition requiring modulating splicing of Usher of an individual in need thereof, said method comprising contacting a cell of said individual with the set of antisense oligonucleotides for skipping of exon 30 and 31 according to claim 1.

14. (canceled)

15. (canceled)

16. The set of antisense oligonucleotides for skipping of exon 30 and 31 according to claim 1, wherein the antisense oligonucleotides bind to and/or are complementary to a polynucleotide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4.

17. The set of antisense oligonucleotides for skipping of exon 30 and 31 according to claim 1, wherein the antisense oligonucleotides bind to and/or are complementary to a polynucleotide selected from the group consisting of SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO: 8.

18. The set of antisense oligonucleotides for skipping of exon 30 and 31 according to claim 1, wherein the antisense oligonucleotides bind to and/or are complementary to a polynucleotide selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO:11 and SEQ ID NO: 12 or a part thereof.

19. The method according to claim 13, wherein the USH2A related disease or condition is USH2A-associated Retinitis pigmentosa (RP).

20. The pharmaceutical composition according to claim 8, wherein the composition is dosed in an amount ranged from 0.05 mg and 30 mg of total antisense oligonucleotides