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Antisense Oligonucleotide (AON)-based Therapy for Leber Congenital Amaurosis Caused by a Frequent Mutation in *CEP290*

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Leber congenital amaurosis (LCA) is the most severe form of inherited retinal degeneration, with an onset in the first year of life. The most frequent mutation that causes LCA, present in at least 10% of individuals with LCA from North-American and Northern-European descent, is an intronic mutation in *CEP290* that results in the inclusion of an aberrant exon in the *CEP290* mRNA. Here, we describe a genetic therapy approach that is based on antisense oligonucleotides (AONs), small RNA molecules that are able to redirect normal splicing of aberrantly processed pre-mRNA. Immortalized lymphoblastoid cells of individuals with LCA homozygously carrying the intronic *CEP290* mutation were transfected with several AONs that target the aberrant exon that is incorporated in the mutant *CEP290* mRNA. Subsequent RNA isolation and reverse transcription-PCR analysis revealed that a number of AONs were capable of almost fully redirecting normal *CEP290* splicing, in a dose-dependent manner. Other AONs however, displayed no effect on *CEP290* splicing at all, indicating that the rescue of aberrant *CEP290* splicing shows a high degree of sequence specificity. Together, our data show that AON-based therapy is a promising therapeutic approach for *CEP290*-associated LCA that warrants future research in animal models to develop a cure for this blinding disease.
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Introduction

Leber congenital amaurosis (LCA) is the most severe form of inherited retinal dystrophy, with an onset of disease symptoms in the first years of life[1] and an estimated prevalence of ~1 in 50,000 worldwide.[2,3] Genetically, LCA is a heterogeneous disease, with 15 genes identified to date in which mutations are causative for LCA.[4,5] The most frequently mutated LCA gene is *CEP290*, accounting for ~15% of all cases.[3,4,6,7] Severe mutations in *CEP290* have been reported to cause a spectrum of systemic diseases that, besides retinal dystrophy, are characterized by brain defects, kidney malformations, polydactyly, and/or obesity.[8–11] There is no clear-cut genotype–phenotype correlation between the combination of *CEP290* mutations and the associated phenotypes, but individuals with LCA and early onset retinal dystrophy very often carry hypomorphic alleles.[3,6,7,12,13] The by far most frequently occurring hypomorphic *CEP290* mutation, especially in European countries and in the United States, is a change in intron 26 of *CEP290* (c.2991+1655A>G).[3,6,7,12] This mutation creates a cryptic splice donor site in intron 26 which results in the inclusion of an aberrant exon of 128 bp in the mutant *CEP290* mRNA, and inserts a premature stop codon (p.C998X). Alternative splicing of the cryptic exon into the mRNA occurs in some, but not all, mRNA transcripts, explaining the hypomorphic nature of this mutation.[6]

LCA, and other retinal dystrophies, for long have been considered incurable diseases. However, the first phase I/II clinical trials using gene augmentation therapy have led to promising results in a selected group of adult individuals with LCA or early onset RP with mutations in the *RPE65* gene.[14–17] Unilateral subretinal injections of adeno-associated virus particles carrying constructs encoding the wild-type *RPE65* cDNA were shown to be safe and moderately effective in some individuals, without causing any adverse effects. In a follow-up study using younger subjects, visual improvements were more sustained, especially in the children who all gained ambulatory vision.[18] Together, these studies have shown the potential to treat LCA, and thereby enormously boosted the development of therapeutic strategies for other genetic subtypes of retinal dystrophies.[19] However, due to the tremendous variety in gene size and technical limitations of the vehicles that are used to deliver therapeutic constructs, gene augmentation therapy may not be applicable to all genes.

An alternative therapeutic strategy depends on the use of antisense oligonucleotides (AONs) that are able to interfere with splicing, and include DNA or RNA molecules/analogues, that can be delivered either as naked oligonucleotides or

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expressed by viral vectors. Although not applicable to all mutations, AONs are ideally suited to block aberrant splice events caused by the activation of cryptic splice sites, and hence restore normal splicing. Here, we describe the use of AONs to redirect normal splicing of *CEP290* in patient-derived lymphoblast cells, and show a sequence-specific and dose-dependent decrease in levels of aberrantly spliced *CEP290* that is accompanied by an increase in correctly spliced *CEP290* mRNA, thereby revealing the potential of AON-based therapy to treat *CEP290*-associated LCA.

Results

The intronic *CEP290* mutation (c.2991+1655A>G) creates a splice donor site that results in the inclusion of an aberrant exon into the *CEP290* mRNA (**Figure 1a** and **b**). Addition of AONs directed against the aberrant exon would prevent the insertion of this exon by preventing the binding of factors that are essential for splicing such as the U1- and U2snRNP complexes, and serine-arginine rich proteins, thereby restoring normal *CEP290* splicing and protein synthesis (**Figure 1c**). AONs can target splice sites as well as exonic sequences, although in the particular case of the Duchenne muscular dystrophy (*DMD*) gene, AONs targeting exonic regions tend to outperform those that target the splice sites.[20] In addition, previous studies have suggested a positive correlation between the capability of AONs to induce exon skipping and the presence of predicted SC35 splice factor binding sites in the target sequence.[21] To design an AON with high exon-skipping potential, the aberrant *CEP290* exon (128 nucleotides exonic sequence plus 15 nucleotides of intronic sequence on each side) was scrutinized for exonic splice enhancer-binding motifs, using the ESE finder 3.0

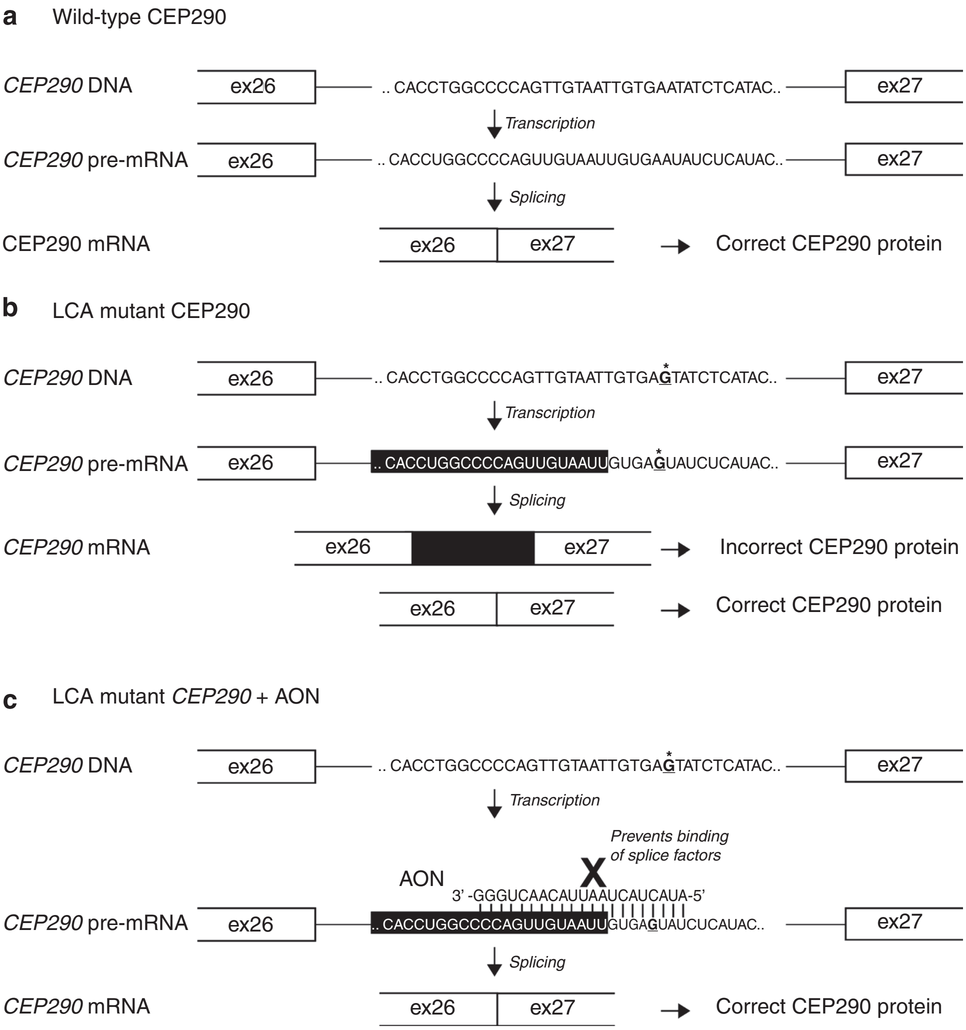
a
Wild-type CEP290
CEP290 DNA
ex26
.. CACCTGGCCCCAGTTGTAATTGTGAATATCTCATAC..
ex27
Transcription
CEP290 pre-mRNA
ex26
.. CACCUGGCCCCAGUUGUAAUUGUGAAUAUCUCAUAC..
ex27
Splicing
CEP290 mRNA
ex26
ex27
Correct CEP290 protein
b
LCA mutant CEP290
CEP290 DNA
ex26
.. CACCTGGCCCCAGTTGTAATTGTGAGTATCTCATAC..
ex27
Transcription
CEP290 pre-mRNA
ex26
.. CACCUGGCCCCAGUUGUAAUUGUGAGUAUCUCAUAC..
ex27
Splicing
CEP290 mRNA
ex26
ex27
Incorrect CEP290 protein
ex26
ex27
Correct CEP290 protein
c
LCA mutant CEP290 + AON
CEP290 DNA
ex26
.. CACCTGGCCCCAGTTGTAATTGTGAGTATCTCATAC..
ex27
Transcription
Prevents binding of splice factors
X
AON
3' -GGGUCAACAUUAAUCAUCAUA-5'
CEP290 pre-mRNA
ex26
.. CACCUGGCCCCAGUUGUAAUUGUGAGUAUCUCAUAC..
ex27
Splicing
CEP290 mRNA
ex26
ex27
Correct CEP290 protein

Figure 1 ***CEP290*** **splicing and antisense oligonucleotide (AON) function.** (**a**) Normal *CEP290* mRNA splicing of exons 26 and 27, resulting in wild-type CEP290 protein. (**b**) The most frequent Leber congenital amaurosis (LCA)-causing mutation is an A-to-G transition (underlined and indicated with an asterisk) in intron 26 of *CEP290*. This mutation creates a splice donor site, which results in the inclusion of an aberrant exon to part of the *CEP290* mRNA and subsequent premature termination of the CEP290 protein. (**c**) Upon binding of sequence-specific AONs, factors involved in splicing will not recognize the aberrant splice donor site in intron 26, resulting in redirection of normal CEP290 splicing and synthesis of a full-length *CEP290* protein.

program.[22] At the 3′-end of the aberrant exon, two SC35-binding motifs were predicted (data not shown). Hence, the first AON was designed such that it encompassed these two motifs (designated AON-3).

To determine whether AON-3 has exon-skipping potential *in vitro*, immortalized lymphoblastoid cells of two unrelated individuals with LCA homozygously carrying the intronic *CEP290* founder mutation c.2991+1655A>G, as well as one control individual, were cultured in the absence or presence of 1 µmol/l AON-3. As expected, in the control individual, only a band representing correctly spliced *CEP290* was observed, whereas in both affected individuals two products were present, one representing correctly spliced, and one representing aberrantly spliced *CEP290* mRNA. Upon addition of AON-3, a strong decrease in aberrantly spliced *CEP290* was noted, in both individuals with LCA (**Figure 2a**). Next, the specificity of AON-3 was assessed by transfecting a sense oligonucleotide directed to the same target site (SON-3). Reverse transcription-PCR analysis showed that in the cells transfected with SON-3, both the aberrantly spliced and the correctly spliced *CEP290* mRNA molecules are still present (**Figure 2b**, left panel), demonstrating the specificity of the antisense sequence. Using an additional pair of primers that amplifies larger products, similar results were obtained (**Figure 2b**, right panel). Interestingly, the decrease in aberrantly spliced *CEP290* appears to coincide with an increased intensity of the product representing correctly spliced *CEP290* mRNA. These data indicate that the aberrant product is not degraded, but that the AON transfection truly induces exon skipping, resulting in the synthesis of more correctly spliced wild-type *CEP290* mRNA. To determine the effective dose of AON-3, cells were transfected with various concentrations of AON-3, ranging from 0.01 to 1.0 µmol/l. Even at the lowest concentration of 0.01 µmol/l, a marked reduction in aberrantly spliced *CEP290* was observed. The maximum amount of exon skipping was observed at 0.05 or 0.1 µmol/l of AON, indicating that these concentrations are sufficient to convert almost all aberrantly spliced *CEP290* (**Figure 2c**).

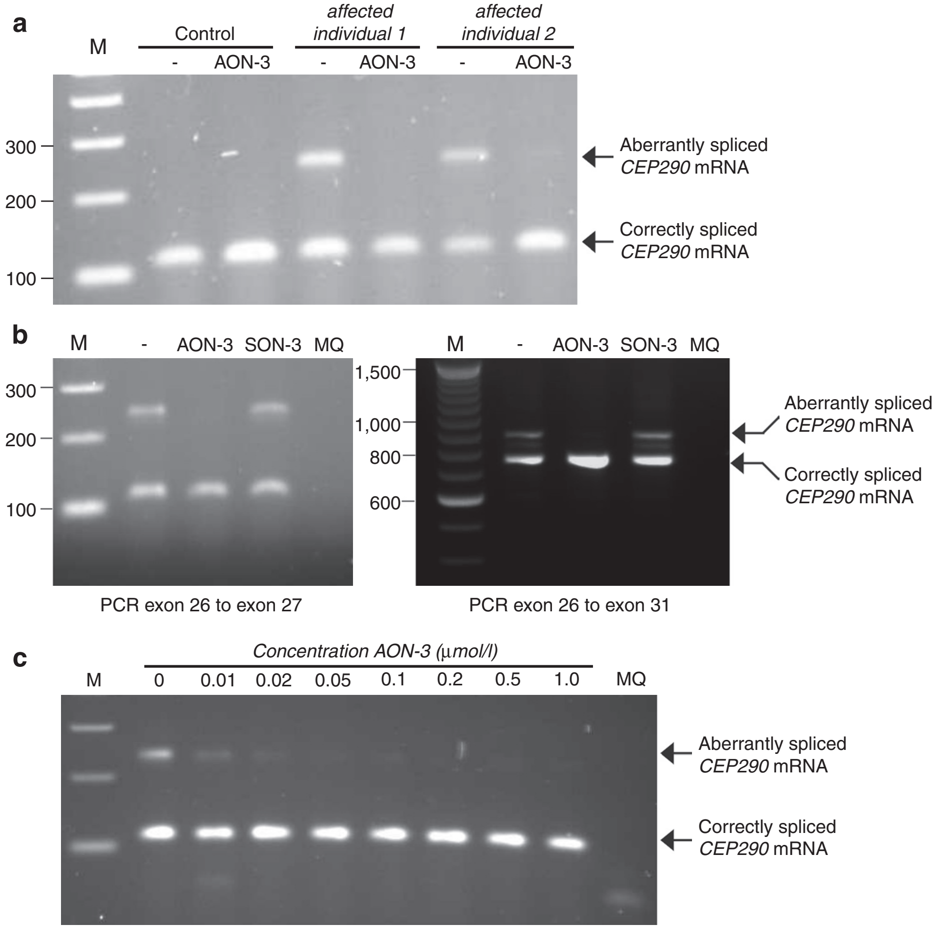
a
M
Control
affected individual 1
affected individual 2
-
AON-3
-
AON-3
-
AON-3
300
200
100
Aberrantly spliced *CEP290* mRNA
Correctly spliced *CEP290* mRNA
b
M
-
AON-3
SON-3
MQ
300
200
100
M
-
AON-3
SON-3
MQ
1,500
1,000
800
600
Aberrantly spliced *CEP290* mRNA
Correctly spliced *CEP290* mRNA
PCR exon 26 to exon 27
PCR exon 26 to exon 31
c
Concentration AON-3 (µmol/l)
M
0
0.01
0.02
0.05
0.1
0.2
0.5
1.0
MQ
Aberrantly spliced *CEP290* mRNA
Correctly spliced *CEP290* mRNA

Figure 2 Antisense oligonucleotide (AON)-based rescue of aberrant CEP290 splicing. (**a**) Reverse transcription-PCR (RT-PCR) analysis of *CEP290* mRNA isolated from lymphoblastoid cells of one control individual and two individuals affected with Leber congenital amaurosis (LCA), that were cultured in the absence or presence of a selected AON (AON-3) directed against the aberrant *CEP290* exon in a final concentration of 1.0 µmol/l. The upper band represents the aberrant *CEP290* splice product, whereas the lower band represents the wild-type *CEP290* splice product. M, 100-bp marker. MQ, negative water control. (**b**) Specificity of AON-based rescue. Similar to (**a**), cells were transfected with AON-3, or a sense oligonucleotide directed to the same target site (SON-3). Left panel: RT-PCR using primers located in exon 26 and exon 27. Right panel: RT-PCR using primers located in exon 26 and exon 31. (**c**) Dose-dependent rescue of *CEP290* mRNA splicing. Similar to (**a**), cells were transfected with different concentrations of the selected AON, ranging from 0.01 to 1.0 µmol/l.

The effectiveness of AONs in splice modulation is thought to depend merely on the accessibility of the target mRNA molecule, and hence may differ tremendously between neighboring sequences. To determine whether this sequence specificity also applies for *CEP290*, several AONs were designed that target the aberrant *CEP290* exon (**Table 1**). This exon consists of 128 base pairs, the majority of which are part of an Alu repeat, one of the most frequent repetitive elements in the human genome,[23] covering the entire 5′-end of the aberrant exon (**Figure 3a**). Hence, the majority of AONs were designed to be complementary to the 3′-end of the aberrant exon or the splice donor site (**Figure 3a**). In total, five AONs were transfected at a final concentration of 0.1 µmol/l, which was shown to be optimal for AON-3. Besides AON-3, also AON-2 and AON-4 resulted in high levels of exon skipping. In contrast, AON-1 that targets the Alu repeat region, and AON-5 that is directed against the splice donor site, hardly showed any exon-skipping potential (**Figure 3b**). This sequence-specific effect is likely caused by secondary structures of the target mRNA that prevent the binding of AONs. Together, our data demonstrate the sequence specificity in AON-based exon skipping of *CEP290* and highlight a small region of the aberrant *CEP290* exon as a potential therapeutic target.

Table 1 Antisense oligonucleotide sequences

RNA oligonucleotide	*Sequence (5′→3′)*	T_m *(°C)*	*Target in aberrant* **CEP290** *exon (nt)*
AON-1	TAATCCCAGCACTTTAGGAG	58	72–91
AON-2	GGGCCAGGTGCGGTGG	58	102–117
AON-3	AACTGGGGCCAGGTGCG	58	106–122
AON-4	TACAACTGGGGCCAGGTG	58	108–125
AON-5	ACTCACAATTACAACTGGGG	58	115–128+6
SON-3	CGCACCTGGCCCCAGTT	58	Nontargeting

Abbreviation: AON, antisense oligonucleotide.
Overview of the sequences of the AONs used in this study. The right column depicts the position of the target sequence in the aberrant *CEP290* exon. AON-5 is directed against the 14 most 3′-exonic nucleotides and the first six nucleotides of the splice donor site. SON-3 is complementary to AON-3 and thus does not target the aberrant *CEP290* exon.

Discussion

In this study, we explored the therapeutic potential of AONs to correct a splice defect caused by an intronic mutation in *CEP290*. In immortalized lymphoblast cells of individuals with LCA homozygously carrying the intronic *CEP290* mutation c.2991+1655A>G, transfection of some but not all AONs resulted in skipping of the aberrant exon, thereby almost fully restoring normal *CEP290* splicing.

AONs have been the focus of therapeutic research for over a decade, for the treatment of a variety of genetic diseases.[24] These strategies include the use of AONs to block the recognition of aberrant splice sites, to alter the ratio between two naturally occurring splice isoforms, to induce skipping of exons that contain protein-truncating mutations, or to induce the skipping of exons in order to restore the reading-frame of a transcript that is disrupted by a genomic deletion, which allows the synthesis of a (partially) functional protein.[24] The latter approach is already being applied in phase I/II clinical trials for the treatment of individuals affected with Duchenne muscular dystrophy, with promising results.[25,26]

The intronic *CEP290* mutation is an ideal target for AON-based therapy, since this mutation results in the inclusion of an aberrant exon in the *CEP290* mRNA which is normally not transcribed. Inducing skipping of this aberrant exon by

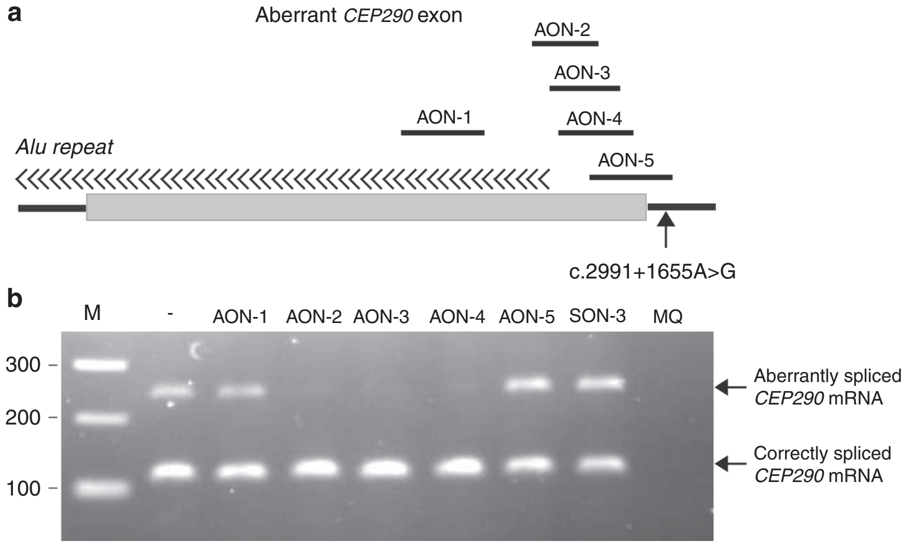

Figure 3 Sequence specificity in antisense oligonucleotide (AON)-based rescue of aberrant *CEP290* splicing. (**a**) Overview of the aberrant *CEP290* exon, and the relative positions of the AONs that were selected. The 5′-end of the aberrant exon is part of an Alu repeat. (**b**) Reverse transcription-PCR (RT-PCR) analysis of *CEP290* mRNA isolated from lymphoblastoid cells of an individual with Leber congenital amaurosis (LCA) that were cultured in the absence or presence of different AONs directed against the aberrant *CEP290* exon (AON-1 to AON-5), or one sense oligonucleotide (SON-3). The AONs and SON were transfected in a final concentration of 0.1 µmol/l. The upper band represents the aberrant *CEP290* splice product, whereas the lower band represents the wild-type *CEP290* splice product. M, 100-bp marker. MQ, negative water control.

AONs appears to almost fully restore the normal *CEP290* mRNA, allowing normal levels of CEP290 protein to be synthesized. A similar approach has been successful in individuals with Duchenne or Becker muscular dystrophy carrying intronic mutations in *DMD*, where addition of AONs resulted in the generation of wild-type dystrophin protein.[27] A second major advantage is that although this AON-approach is a mutation-specific therapeutic strategy, the intronic *CEP290* mutation is by far the most frequent LCA-causing mutation.[4] Based on the estimated prevalence of LCA (1:50,000), and the observed frequency of the intronic *CEP290* mutation in Northern-Europe (26%)[28] and the United States (10%),[3] at least one thousand and, depending on the frequency of the mutation in other populations, perhaps many more individuals worldwide have LCA due to this mutation. Finally, although the LCA phenotype associated with *CEP290* mutations is severe, it appears that the photoreceptor integrity, especially in the macula, as well as the anatomical structure of the visual connections to the brain, are relatively spared in individuals with LCA carrying *CEP290* mutations, which would allow a window of opportunity for therapeutic intervention.[29]

The study described here provides a proof-of-principle of AON-based therapy for *CEP290*-associated LCA *in vitro*, using immortalized lymphoblast cells from affected individuals. In order to determine the true therapeutic potential of this method for treating LCA, additional studies are needed that include the development of therapeutic vectors, and assessment of efficacy and safety in animal models. AONs can be delivered in two ways, either as naked molecules (or conjugated to cell-penetrating peptides), or via viral vectors. A single injection of naked AONs has recently been shown to be effective for several months in a mouse model for spinal muscular atrophy.[30] Naked AONs can be delivered to the retina by intraocular injections, although in each individual multiple injections would still be required. Alternatively, by using viral vectors, a single subretinal injection would suffice to allow a long-term expression of the therapeutic construct. Previously, others have used recombinant adeno-associated viral (rAAV) vectors carrying U1- or modified U7snRNA constructs to efficiently deliver AON sequences, in the *mdx* mouse model for DMD, or in myoblasts from individuals with DMD, respectively.[31,32] In line with this, AONs targeting the aberrant exon of *CEP290* could be cloned into such constructs, and delivered to the retina by subretinal injections of rAAV-5 or -8 serotypes that efficiently transduce photoreceptor cells where the endogenous *CEP290* gene is expressed.[33,34] Using rAAV-2 vectors, no deleterious immune response was evoked upon subretinal injections of these vectors in individuals with *RPE65* mutations,[35] and also for rAAV-5 and rAAV-8, immune responses appear to be benign, at least in animal models.[36,37] One final safety aspect concerns the specificity of the sequence that is used to block the splicing of the aberrant *CEP290* exon. As stated before, the majority of this exon is part of an Alu repeat, and AONs directed against this repeat will likely bind at multiple sites in the human genome, increasing the chance to induce off-target effects. The AONs that were shown to be effective in this study do not fully target the Alu repeat sequence, but are also not completely unique in the human genome. However, when blasting against the EST database, no exact hits are found, indicating that at the level of expressed genes, these sequences are unlikely to induce off-target effects and deregulate normal splicing of other genes. To further study the efficacy and safety of AON-based therapy for *CEP290*-associated LCA *in vivo*, including the use of AON expression driven by viral vectors, we are currently generating a transgenic knock-in mouse model that carries part of the human *CEP290* gene (exon 26 to exon 27, with and without the intronic mutation) which is exchanged with its mouse counterpart.

Compared to gene augmentation therapy, AON-based therapy has a number of advantages. First, in gene augmentation therapy, a ubiquitous or tissue-specific promoter is used to drive expression of the wild-type cDNA encoding the protein that is mutated in a certain individual. For instance in one clinical trial for *RPE65* gene therapy, the chicken beta-actin promoter was used.[17] Using either constitutive promoters or fragments of the endogenous promoters, it is difficult to precisely control the levels of expression of the therapeutic gene. In some cases, like for the RPE65 protein that has an enzymatic function, expression levels beyond those of the endogenous gene might not be harmful to the retina. For other genes however, including those that encode structural proteins like CEP290, tightly-regulated expression levels might be crucial for cell survival, and overexpression of the therapeutic protein might exert toxic effects. Using AONs, the therapeutic intervention occurs at the pre-mRNA level, and hence does not interfere with the endogenous expression levels of the target gene. A second issue is the use of the viral vector. Of a variety of different recombinant viral vectors, rAAVs are considered to be most suitable for treating retinal dystrophies, because of their relatively high transduction efficiency of retinal cells, and their limited immunogenicity. The major drawback of rAAVs however is their limited cargo size of 4.8 kb. Again, for some genes like *RPE65*, this is not a problem. For many other retinal genes however, including *CEP290* (with an open reading frame of 7.4 kb), but also *ABCA4* and *USH2A*, the size of their full-length cDNAs exceeds the cargo size of the currently available pool of rAAVs. One way to overcome this problem is to express cDNAs that express only partial proteins with residual activity, as has been suggested for CEP290 in two studies employing the zebrafish model, either by expressing the N-terminal region of the protein[38] or a 299-amino acid domain of CEP290 that is deleted in the *rd16* mouse.[39] Other viral vectors, like lentivirus or adenoviruses have a higher cargo capacity than rAAVs (~8 kb), but are less efficient in transducing retinal cells, and in addition, adenovirus has a higher immunogenic potential.[19] For AON-based therapy, the size limitations of AAV are not a problem, since the small size of the AONs and the accompanying constructs easily fit within the available rAAVs.

In conclusion, this study shows that administration of AONs to cultured cells of affected individuals almost fully corrects a splice defect that is caused by a frequent intronic mutation in *CEP290* that causes LCA. These data warrant further research to determine the therapeutic potential of AON-based therapy for *CEP290*-associated LCA, in order to delay or cease the progression of this devastating blinding disease.

Materials and Methods

Design AONs. The 128-bp sequence of the aberrant *CEP290* exon that is included into the mutant *CEP290* mRNA was analyzed for the presence of exonic splice enhancer motifs using the ESE finder 3.0 program (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home). RNA AONs were purchased from Eurogentec, and designed with a T_m of 58°C, and modified with a 2′-O-methyl group at the sugar chain and a phosphorothioate backbone, and dissolved in phosphate-buffered saline.

Cell culture. Human B-lymphoblast cells of individuals affected with LCA and homozygously carrying the intronic mutation in *CEP290* were immortalized by transformation with the Eppstein–Barr virus, as described previously.[40] Cells were cultured in RPMI1640 medium (Sigma, St Louis, MO) containing 10% (vol/vol) fetal calf serum (Sigma), 1% 10 U/µl penicillin and 10 µg/µl streptomycin (Sigma), at a density of 0.5×10^6 cells/ml. Fresh medium was supplied twice a week.

Transfection of AONs. A day before transfection, 1.0×10^6 cells were seeded in each well of a 6-wells plate, in a total volume of 2 ml complete medium. Transfection mixtures were prepared by combining 2.5 µl AON in a desired concentration, or distilled water, 5 µl transfection reagent (ExGen *in vitro* 500; Fermentas, St Leon-Rot, Germany) and 92.5 µl 150 mmol/l NaCl, and incubated at room temperature for 10 minutes, before addition to the cells. Six hours after transfection, 8 ml of low-serum medium (complete medium with only 1% fetal calf serum) was added. Forty-eight hours after transfection, cells were collected and washed with 1× phosphate-buffered saline, before directly proceeding to RNA isolation.

RNA isolation and reverse transcription-PCR. Total RNA was isolated from transfected lymphoblastoid cells using the Nucleospin RNA II isolation kit (Machery Nagel, Düren, Germany), according to manufacturer's protocol. Subsequently, 1 µg of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands). Five percent of the cDNA was used for each PCR. Part of the *CEP290* cDNA was amplified under standard PCR conditions supplemented with 5% Q-solution (Qiagen, Hilden, Germany), and using forward primer 5′-TGCTAAGTACAGGGACATCTTGC-3′ and reverse primer 5′-AGACTCCACTTGTTCTTTTAAGGAG-3′ or 5′-TGGCAATGAGCGACTTTC-3′ that are located in exon 26, exon 27, and exon 31 of the human *CEP290* gene, respectively. PCR products were resolved on a 1.5% agarose gel. Bands presumably representing correctly and aberrantly spliced *CEP290* were excised from the gel, purified using Nucleospin Extract II isolation kit and sequenced from both strands with the ABI PRISM Big Dye Terminator Cycle Sequencing V2.0 Ready Reaction kit and the ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

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