Oligonucleotide-Based Splice Correction of the *ABCA4* c.5461-10T>C Mutation in Stargardt Disease Type 1

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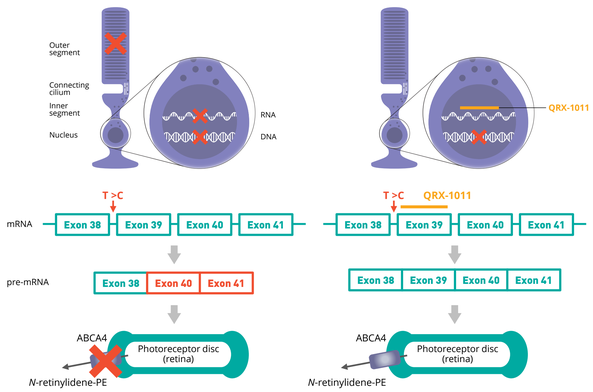
Introduction

- Stargardt disease type 1 (STGD1) is the most common form of inherited macular dystrophy causing progressive impairment of central vision, with onset typically in childhood or young adulthood[1].
- STGD1 has an autosomal recessive mode of inheritance associated with disease-causing mutations in the *ATP-binding cassette, subfamily A, member 4 (ABCA4)* gene, which encodes a transport protein localized in photoreceptor outer segment disk edges.
- ABCA4 mainly functions to remove potentially toxic retinoids, such as N-retinylidene-phosphatidylethanolamine, which originate from the phototransduction process. Failure of this transport, caused by absent or dysfunctional ABCA4, results in accelerated deposition of a major lipofuscin fluorophore, N-retinylidene-N-retinylethanolamine, in the retinal pigment epithelium with subsequent RPE apoptosis and photoreceptor degeneration.
- *ABCA4* c.5461-10T>C is the third most common mutation and causes exclusion of exon 39 in the mRNA, resulting in a shift of the open reading frame and premature stop codons[2,3].



In wild-type photoreceptors, splicing results in correct transcripts and therefore normal functional protein. However, when the c.5461-10T>C mutation is present, the splicing machinery does not recognize exon 39 and as a result this exon is excluded in the mature mRNA which in turn results in a frameshift. This significantly reduces the ABCA4 activity. AONs are designed to bind to *ABCA4* pre-mRNA and enhance the inclusion of exon 39.

Objectives

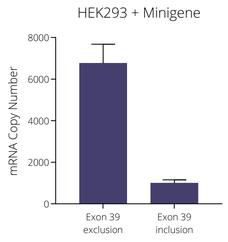
- **Identify AONs correcting the *ABCA4* c.5461-10T>C mediated splice defect**
- **Assess the feasibility of AON mediated splice correction in photoreceptors**

Materials & Methods

ABCA4 minigene was generated as described previously[3]. HEK293 cells were transfected with 50 ng plasmid using maxPEI and after 24 hours with 250 nM AON using Lipofectamine™ 2000. AONs used in this study contain phosphorothioate backbone and 2'-O nucleoside modification. Cells were harvested 24 hours later and *ABCA4* transcripts were quantified using custom designed isoform specific TaqMan® droplet-digital PCR (ddPCR) assays. For *in vivo* studies wild-type C57BL/6 mice received bilateral IVT injection of the AON using a previously published protocol[4]. *Usherin* transcripts levels were quantified using ddPCR. To assess *in vivo* AON delivery, eyes were fixed overnight in Hartmann's fixative and embedded in paraffin. AON was visualized in retina using complementary probe with Cy5 label by *in situ* hybridization. Images were acquired on a LSM800 confocal microscope. Retinal organoids were generated from wild type iPSC as described previously[3].

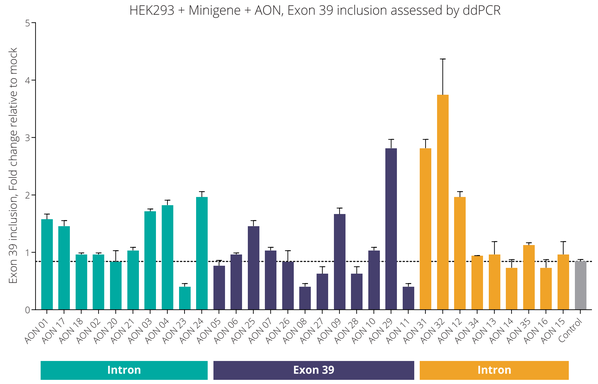
Results

ABCA4 c.5461-10T>C Minigene Displays Defective Exon 39 Splicing

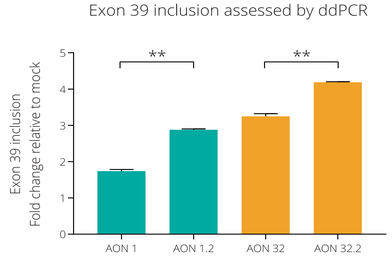


Since *ABCA4* is almost exclusively expressed in the retina, a minigene construct containing *ABCA4* exon 39 along with the flanking introns carrying c.5461-10T>C mutation was used to screen for splice modulating AONs. Splice variant expression of the *ABCA4* exon 39 minigene in HEK293 cells was quantified using ddPCR. Data is shown as mean ± SEM. As expected, most of the transcripts lacked exon 39 owning to the c.5461-10T>C mutation. To correct the splice defect mediated by the c.5461-10T>C mutation, 31 AONs binding to the length of exon 39 (124 bases) and the adjacent introns were designed and tested.

Identification and Optimization of Antisense Oligonucleotides Targeting the c.5461-10T>C Mutation

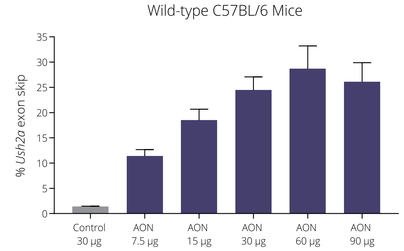


Quantification of the relative fold change of percentage of exon 39 inclusion in HEK293 cells transfected with the *ABCA4* mutant exon 39 minigene and the AONs. Data is shown as mean ± SEM. 'Control' refers to an unrelated non-*ABCA4* annealing AON, levels of which are indicated by the dotted line. As the AONs have overlapping sequences among themselves, many candidates were identified which increased *ABCA4* exon 39 inclusion. Moreover, this analysis revealed designated regions where targeting resulted in increased exon 39 inclusion. For example, AON12, AON31 and AON32 bind to the same overlapping region.



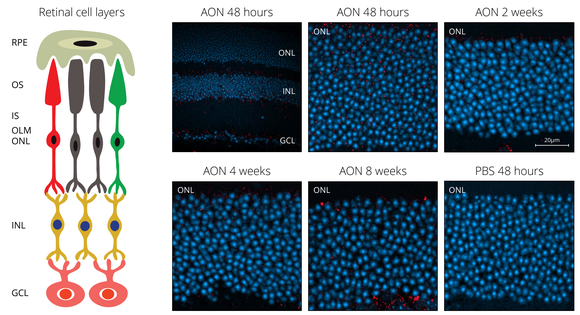
Promising AONs identified in the first screen are currently being optimized further for sequence and chemistry. Results are shown for two AONs, targeting different ABCA4 regions, which showed significant improvement in exon 39 inclusion upon optimization. Data is shown as mean ± SEM. Student's t-test **p < 0.01.

Antisense Oligonucleotide Mediated Splice Modulation in Photoreceptors



Currently there are no animal models for the *ABCA4* c.5461-10T>C mediated splice defect. Hence we assessed the *in vivo* feasibility of AON mediated splice modulation in photoreceptors using a surrogate retinal target, *Ush2a*, in mice. Wild-type C57BL/6 mice were treated with a single intravitreal injection of 7.5 to 90 µg of exon 12 skipping oligo per eye and maintained for 7 days. Percentage of *Ush2a* exon skip was assessed using quantitative ddPCR. Results show that the splice modulating AON resulted in dose-dependent exon skip *in vivo*. Since *Ush2a* is a photoreceptor specific gene these findings demonstrate proof-of-concept of the ability of AONs to reach photoreceptor nuclei and modulate splicing.

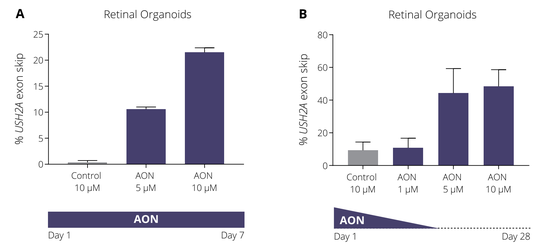
In vivo Uptake and Retention of Antisense Oligonucleotides in the Outer Nuclear Layer of the Retina



To confirm the delivery, we have visualized the AON in the retinal outer nuclear layer (ONL), which comprises of photoreceptor nuclei, using *in situ* hybridization. Wild-type C57BL/6 mice received a single bilateral 1µl intravitreal injection of 30 µg of AON and were maintained for 2, 14, 28, or 56 days. Clear AON signal was detected in the ONL at all time points tested here confirming delivery and indicating long half-life. No signal was detected in the PBS treated retina.

Antisense Oligonucleotide Mediated Splice Modulation in Human Retinal Organoids

Generating animal models for splicing defects represents a challenge since human splicing mutations may not be recognized by the splicing machinery of other species[5]. In the absence of animal models, patient iPSC derived 3D retinal organoids provide an excellent platform to test therapeutic interventions[6]. In addition, they simulate the disease phenotype and provide an appropriate cellular model with the genetic mutations in genomic context.



AON with similar sequence and chemistry to that of mouse *Ush2a* targeting AON was tested in human iPSC derived retinal organoids. Wild-type iPSC were differentiated into retinal organoids for 60 days and were treated with *Ush2a* exon 13 (equivalent of mouse *Ush2a* exon 12) skipping AON either for 7 days continuously (A) or for 28 days mimicking a half-life of 2 days (B), and the effect was measured after 7 or 28 days, respectively. The AON resulted in a dose-dependent exon skip (A) in optic cups and the effect is similar to what was found *in vivo*. Interestingly, significant amount of exon 13 skip was noticed even after 2 weeks of removing all the AON from the medium (B), indicating long lasting effect of AON after treatment.

Conclusion

In this study we:

- **identified AONs that can correct the *ABCA4* c.5461-10T>C mediated splice defect *in vitro*;**
- **demonstrated the *in vivo* splice modulating activity of AONs using a surrogate retinal target, Ush2a, in photoreceptors;**
- **observed that AONs have long retinal half-life that might allow monthly or quarterly dosing;**
- **noticed similar splice modulating activity of AON in retinal organoids which represent a promising test system in the absence of an animal model.**

Taken together, splice-correcting antisense oligonucleotides represent a promising treatment option for STGD1.

A patent application claiming the invention as disclosed in this poster was filed (PCT/EP2018/059542).

References

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