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Retina Australia Research Report 2023

Project Title: Establishing novel AAV gene editing for Usher syndrome

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Project synopsis: Eye diseases caused by genetic defects are numerous and devastating to patients and families. These conditions affect the neural retina at the back of the eye in particular the light sensing cells, the photoreceptor cells. The aim of this project is to establish proof-of-concept for adeno associated viral vectors (AAV) gene editing (CRISPR-Cas9) treatments designed specifically for genetically confirmed Usher Syndrome subtype 1 (Usher1b and 1f subtypes) Australian patients. These were tested in patient derived retinal organoids to assess for treatment efficacy. We started to investigate the use of Homology Independent Targeted Integration (HITI) gene editing, which was delivered using our well-established gene therapy platform for organoids together with novel lipid nanoparticle encapsulating messenger RNA (LNP) for Cas9 protein and a single guide RNA (sgRNAs) targeting Usher1, MYO7a and PCDH15 genes.

Scientific Outcomes:

Aim 1: Designing and evaluating AAV and LNP gene editing approaches in Usher1 subtype 1b and 1f retinal organoids
Hypothesis 1: A combinatory treatment using AAV templates and Cas9 LNPs can efficiently edit the genome and promote gene integration.

Aim 2: To demonstrate efficacy of AAV gene editing to rescue disease biomarkers
Hypothesis 2: HITI gene editing can rescue disease specific phenotypes in retinal organoids

Results 1. Differentiation of retinal organoids

We first optimised the differentiation of retinal organoid differentiation protocol. The efficiency of these protocols vary considerably between different cell lines, therefore we examined using qPCR the endogenous levels of BMP4 and FGF2 in each cell line to understand which cell line had the potential generate forebrain regions and therefore form retinal organoids. Following this initial characterisation we successfully generated retinal organoids using all the cell lines. Usher1b and Usher1f retinal organoids developed normally and contained photoreceptor cells and other retinal cell types.

Results 2 Finalising AAV gene editing constructs and preliminary LNP validation

We designed our viral vector constructs for the HITI CRISPR editing approach for Usher1b and Usher1f. For Usher1b, the number of exons included in the AAV construct is limited to exons 28-49 (AAV.HITI template). This design, encompassing exons 28-49, targeted the large intron between exons 27 and 28 which provides multiple targets for guide sequence selection. Two of our lines can be treated with this construct. For Usher1f, both our patients have mutations on the first exons and therefore the construct encompassed exons 1-27.

We designed five different guide RNAs and tested their efficiency in HEK cells first. From this, we were able to narrow it down to two guide RNAs pairs. Two AAV constructs each containing a pair of guides flaking the template sequence were cloned made into AAV 7m8 vectors for retinal organoids transduction.

Results 3 Testing optimal AAV/LNP gene editing treatments

First we evaluated cutting efficiency, i.e formation of indels, following AAV2.7m8.RK.SaCas9.U6gRNA transduction in 17-20 weeks retinal organoids. Organoids were collected four weeks post-transduction and DNA sequencing was performed to check for cutting efficiency. It was evident that one of the selected guides outperformed the other. Robust cutting efficiency of 20% was observed in treated retinal organoids with the most efficient guide. In parallel Myosin7a protein levels were characterised using immunohistochemistry and western blot showing a decrease in patient organoids. This biomarker will be crucial to establish if rescue of protein will be achieved once combinatory AAV2.7m8.RK.SaCas9.U6gRNA and AAV2.7m8.template are performed.

In parallel, we also tested the delivery of LNPs to retinal organoids using a LNP containing GFP and luciferase mRNA. Luciferase LPNs expression could be determined using a plate reader while GFP mRNA was not detected. Different concentrations of LPNs were added to 15 weeks old cultures of retinal organoids. The successful delivery and luciferase expression demonstrated that lipid nanoparticles will allow entry of the gene therapy into the retina, without long term expression of the Cas9.

Results 4. Specific functional assays to assess the treatment of Usher1b

Before testing if the gene therapy can rescue disease biomarkers, we needed to establish specific biomarkers and assays that can be used to test the rescue of functionality. As mentioned above we utilised qPCR, immunohistochemistry and western blots to investigate the disease effects on MYO7A and PCDH15 in usher1b and usher1f, respectively, in the retinal organoids. We initially found that MYO7A and PCDH15, were downregulated at the RNA and protein level. Additionally, within these retinal organoids, we found that oxidative stress is prevalent, and we optimised specific mitochondrial assays to identify disease rescue. Using these markers, we can now continue to test the rescue of disease in our gene therapy trials.