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Engineering human retinal organoids to study and treat autosomal dominant cone-rod dystrophies

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1. Main Text

Cone-rod dystrophy (CORD) is a debilitating inherited retinal disease affecting 1 in 20,000 – 100,000 individuals. It leads to progressive loss of central vision, impaired colour perception, and photophobia [1]. Severe autosomal dominant forms of CORD (adCORD) are associated with more than 20 mutations in the *GUCA1A* gene, which encodes GCAP1 (guanylate cyclase-activating protein 1), a protein essential for photoreceptor's function and viability. Among these, a missense variant with autosomal dominant inheritance pattern (c.332A>T; p.(Glu111Val); E111V) is linked to a particularly severe clinical phenotype and represents an ideal model for developing a variant-independent therapy for *GUCA1A*-associated adCORD [2]. To date, there is no treatment option for adCORD.

Accurate disease models would be essential for both mechanistic characterisation and therapy fine tuning. Both *in vivo* and *ex vivo* animal models are used to shed light on the disease, mainly organotypic murine [3] and porcine retinal cultures [4]. These represent a valid model of the disease, however with some limitations which hinder the development of clinically applicable treatments.

In the last decade human retinal organoids have been produced and characterized, thus providing a more human-like, longer-lasting, reliable, reproducible, and controllable model [5, 6]. This project aims to establish an in vitro human retinal organoid model system, starting from human induced pluripotent stem cells (hiPSCs) and performing genome editing via CRISPR-Cas9 to study mutation-specific adCORD phenotypes, such as E111V, allowing us to visualize the initiation and progression of disease phenotypes as the retina develops. This model can then be exploited for the characterisation and treatment options investigation of adCORD by testing protein-loaded nanoparticles. In particular, liposomes with a lipid composition similar to rod outer segment membranes [7] will be first loaded with a reporter protein, mCardinal2, to assess liposomes' ability to deliver their cargo to photoreceptors in the RO, and secondly with GCAP1 as an attempt to rescue/induce the E111V phenotype.

Overall, this study will provide a mechanistic understanding of the retina-specific phenotypes in adCORD patients and potential strategies for therapeutic intervention without neither patient recruitment nor use of animal models.

2. Methods and results

Cas9 enzyme carrying a nuclear localization signal (NLS) at the N-terminus and a 6xHis-tag at the C-terminus was expressed in *E.coli* and purified in two-step chromatography, *i.e.* IMAC and IEX, and shortly characterized in terms of purity (SDS-PAGE), secondary structure (far-UV circular dichroism). Its activity was then assessed *in vitro* following both DNA extraction and HEK293T cells nucleofection, upon incubation of Cas9 with 5 different sgRNAs, specifically designed for the knock-out of *GUCA1A* gene in a region as close as possible to the mutation responsible for the E111V phenotype. As a result, the RNP complex Cas9-sgRNA proved to be effective with all the tested sequences. After analysis of predicted on-target and off-target activity of each sgRNA, two were selected for the nucleofection of hiPSCs, with the addition of a donor DNA carrying two mutations to induce: 1) PAM disruption; 2) the adCORD-causing genotype. After bulk Sanger sequencing of the region of interest, the optimal combination of sgRNA1 and donor DNA HDR1 was selected. After single cell cloning and sequencing, and confirming the presence of the aforementioned mutations, two clones were selected for further differentiation into human retinal organoids (ROs). In particular, one clone had a heterozygous PAM site disruption and a homozygous c.332A>T E111V, whereas in the other clone both mutations were homozygous. In parallel, non-edited isogenic hiPSCs were differentiated into ROs as a control and a tool for later nanoparticles' testing. Two different protocols were followed: The first protocol is adapted from [8] Fligor et al 2020 and [9] Corral-Serrano et al 2023, starting from three-dimensional embryoid bodies (EBs) formation, followed by plating after 7 days and lifting of neuroretinal vesicles (NRVs) on day 21, which were cultured in suspension. The second protocol is adapted from [10] West et al 2022 and [9] Corral-Serrano et al 2023, where differentiation starts in a two-dimensional system and after roughly 3 weeks NRVs are formed, excised, and cultured in suspension with specific retinal differentiation and photoreceptor maturation media. To capture the onset and late stage of the disease model, two time points were selected for adCORD phenotype characterisation, day 150 and day 200. At these time points, ROs were harvested and frozen after fixation with 4% PFA and gelatin/sucrose embedding for further characterisation.

As for rod outer segment (ROS)-like liposomes formulation, lipid aliquots with a fixed molar ratio were prepared in chloroform, and the organic solvent was evaporated in a concentrator connected to a vacuum pump, thus allowing the formation of a thin lipid layer. The molar ratio mimics the composition of ROS membranes, which is the following: 40% phosphatidylcholine, 40% phosphatidylethanolamine, 15% phosphatidylserine and 5% cholesterol. The thin layer was hydrated with a fixed volume of

protein solution in PBS, in particular mCardinal2 or GCAP1, vortexed and sonicated before extrusion through 100 nm diameter filters. Liposomes were then washed in concentrators with a 100,000 MWCO and, after assessing their hydrodynamic diameter and concentration, they were administered to ROs, finally collected and analysed at day 200.

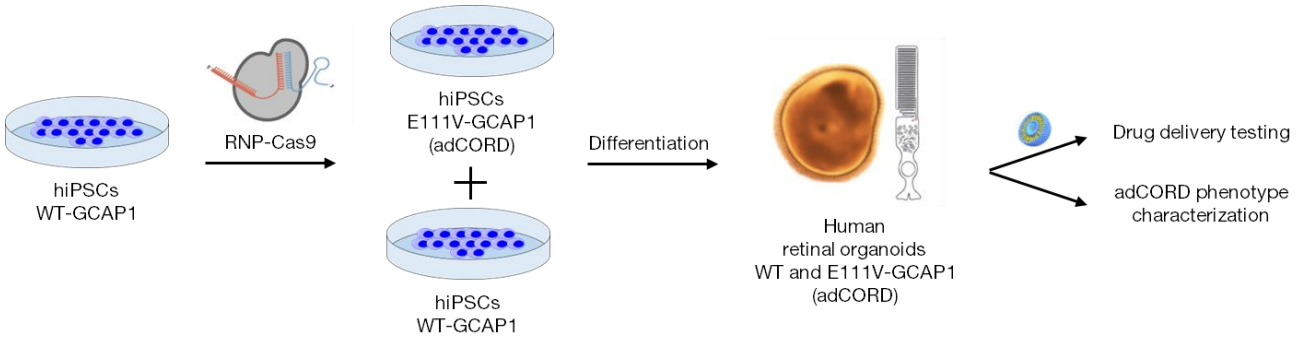

Fig. 1. Graphical abstract. Genome editing and differentiation of hiPSCs into photoreceptors, forming retinal organoids on which morphological characterisation studies of adCORD and drug delivery of lipid nanoparticles loaded with protein, will be performed. Adapted from [6].

3. Acknowledgement

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4. References

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