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RetGC-GUCY2D retinal organoid disease model for AAV gene therapy development

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1. Introduction

Retinal membrane guanylyl cyclase (RetGC-GUCY2D or RetGC), is located in disc membranes of photoreceptor outer segments and is one of the key enzymes in photoreceptor physiology. Mutations in *RetGC-GUCY2D* cause cone-rod dystrophies. The role of RetGC is to replenish cGMP levels after light exposure. In the dark, cGMP levels are sustained at a steady rate, keeping the cGMP-gated channels open and maintaining partial depolarization of the cells by allowing influx of the inward current. Exposure to light leads to cGMP hydrolysis and channel closure, facilitating a sharp decline in intracellular Ca^{2+} and hyperpolarization of the cells.
CRISPR/CAS9 was used to generate *RetGC* Knockout (RetGC KO) and isogenic wildtype control retinal organoids. Following iPSC-retinal organoid differentiation, RetGC KO retinal organoids were compared to the wildtype non-gene edited isogenic control derived from the same cell line. The presence of photoreceptor markers and the absence of RetGC protein was verified by immunostaining, western blot and qPCR.

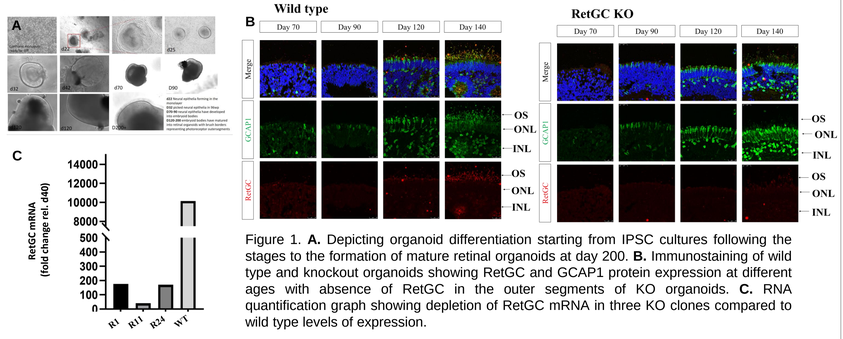
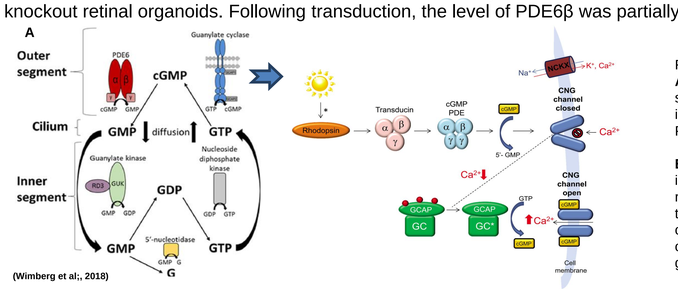
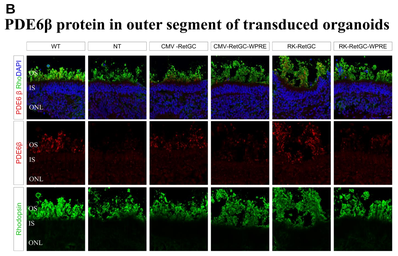


Figure 1. **A.** Depicting organoid differentiation starting from IPSC cultures following the stages to the formation of mature retinal organoids at day 200. **B.** Immunostaining of wild type and knockout organoids showing RetGC and GCAP1 protein expression at different ages with absence of RetGC in the outer segments of KO organoids. **C.** RNA quantification graph showing depletion of RetGC mRNA in three KO clones compared to wild type levels of expression.

3. PDE6β protein expression

PDE6β's role is to inhibit cGMP production which leads to the closure of cGMP channels and consequently to the depletion of calcium ions. Reduction of calcium ions leads to the activation of guanylate cyclase activating proteins (GCAPs) which bind with RetGC to produce cGMP allowing the calcium ions to re-enter and repeat the phototransduction cycle. PDE6β levels were reduced in RetGC knockout retinal organoids. Following transduction, the level of PDE6β was partially restored.





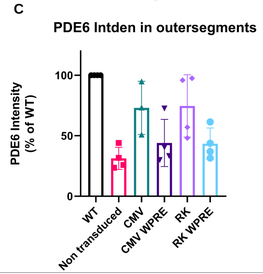


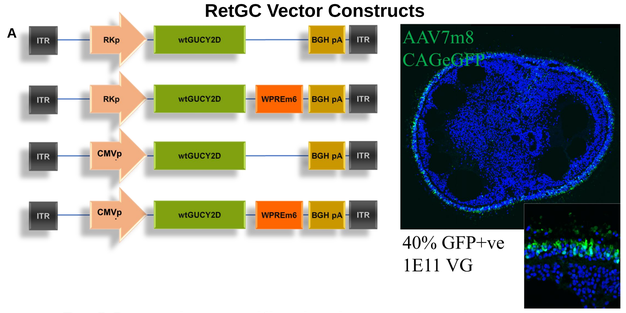
Figure 2.
A. Phototransduction cycle, showing the order of events involving RetGC and PDE6β.

B. PDE6β is downregulated in RetGC KO and partially restored following transduction. Rhodopsin confirms the presence of outer segments. Rhodopsin; green. PDE6β; red.

C. Quantification of PDE6β in outer segments. Following transduction with RetGC viral vectors, PDE6β protein is increased compared with non transduced organoids. 7m8-CMV-RetGC and 7m8-RK-RetGC exhibiting a marked increase in staining compared to 7m8-CMV-RetGC-WPRE and 7m8-RK-RetGC-WPRE.

2. RetGC vector design and retinal organoid transduction

Four AAV vectors were designed to test restoration of RetGC protein in photoreceptors. CMV and Rhodopsin kinase (RK) promoters were compared, as well as the presence or absence of Woodchuck Hepatitis virus post translational regulatory element (WPRE) was assessed in driving expression of a wild-type human *RetGC* gene sequence (wtGUCY2D). All vectors were packaged into 7m8 capsids.



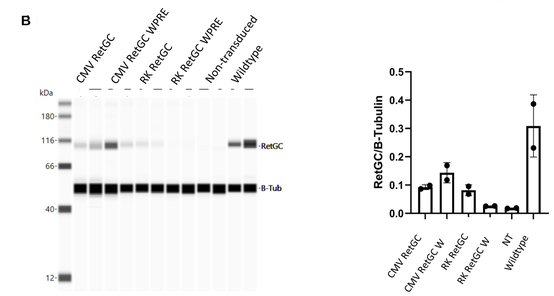


Figure 3. **A.** Design of RetGC viral constructs used to make the viral vectors along side an image showing transduction with 7m8CAGeGFP vector in the organoid photoreceptor layer. **B.** Protein Immunoblotting showing partial restoration of RetGC protein levels.

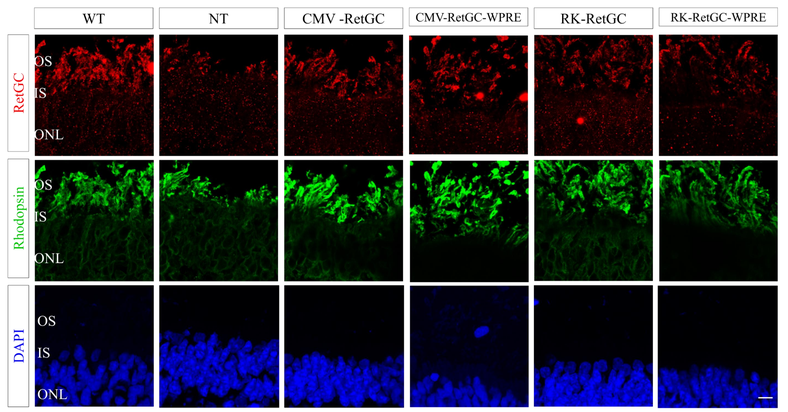
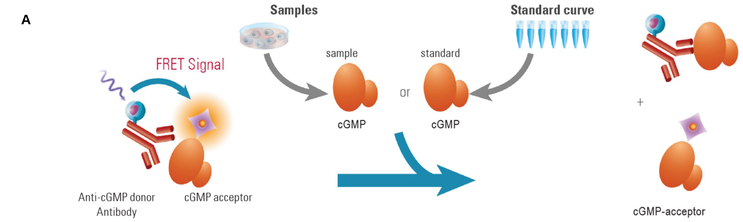
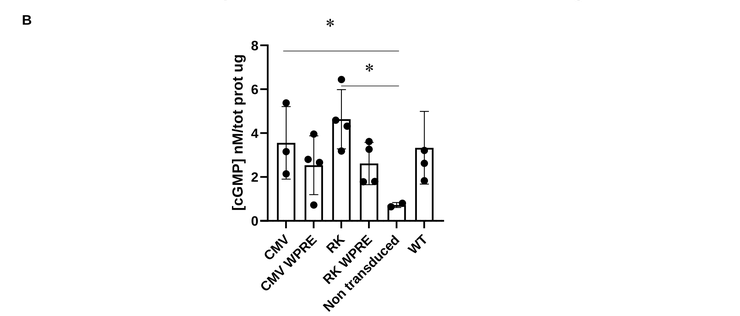


Figure 4. RetGC (red) is expressed in WT retinal organoids and translocated to the photoreceptor outersegment (OS) which is co-stained with OS marker rhodopsin (green). In non-transduced RetGC KO (NT), RetGC protein is absent from the OS despite the formation of rhodopsin positive OS. Organoids following 3 weeks of transduction demonstrate partial rescue of RetGC protein staining which is correctly localized to the OS (scale bar = 10µm).

4. Quantification of cGMP by a FRET-based assay

Following light stimulation cGMP levels in photoreceptors are depleted. In wildtype photoreceptors RetGC enzyme restores cGMP levels in the dark. WT and RetGC KO organoids, transduced and non-transduced with the 7m8 vectors, were exposed to a cycle of light/dark to induce the production of cGMP. Following dissection to isolate the photoreceptors layer and sample lysis, cGMP levels were assayed in the presence of a PDE-inhibitor. cGMP levels (nM) were determined using a FRET-based assay and values were normalised on the total protein amount (µg) per sample.





One Way ANOVA - Kruskal-Wallis test - p=0.0033 RK Vs NT
p=0.048 CMV vs NT

Figure 5. **A.** Schematic of the principle behind the cGMP FRET based assay. When the dyes are in close proximity, the excitation of the donor with a light source triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength. The cGMP present in the sample competes with the binding between the two conjugates and thereby prevents FRET from occurring. The specific signal is inversely proportional to the cGMP concentration.
B. Quantification of cGMP levels. WT, RetGCKO (non transduced) and RetGCKO organoids transduced with 7m8 vectors packaging our 4 vector construct designs were used to quantify cGMP levels at 3 weeks following transduction. The 7m8-CMV-GUCY2D and 7m8-RK-GUCY2D vectors provide statistically significant differences in cGMP production compared to non-transduced RetGCKO organoids.

5. Conclusions

AAV vectors expressing human *RetGC-GUCY2D* were used to transduce human photoreceptors in RetGC KO organoids. RetGC protein was produced by all vector designs with varying efficiency. Overall, the inclusion of WPRE appeared to show a trend towards reduced efficacy as it was assessed by two measurements of vector potency. Both CMV and RK promoter bearing vectors lacking WPRE demonstrated improved PDE6β localisation correction compared to vectors lacking WPRE. Likewise, in a cGMP quantification assay we observed 7m8-CMV.RetGC and 7m8-RK.RetGC restore cGMP levels in transduced organoids comparable to WT organoid levels.
Whilst the AAV2-7m8 serotype is powerful in assessing vector construct efficacy in the human retinal organoid platform, in designing a gene therapy vector for use in patients with *RetGC-GUCY2D* mutations, an alternate serotype needs to be considered. Moreover, further vector construct sequence optimisations would be advantageous to increase RetGC production and maximise the potential benefit in patients.

References:
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