Models of Autosomal Dominant Optic Atrophy (ADOA) using iPSCs and Response to Targeted Augmentation of Nuclear Gene Output (TANGO) Antisense Oligonucleotides (ASOs) Treatment

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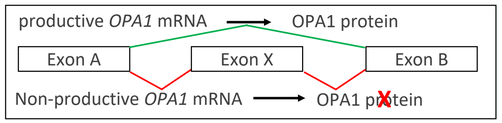
Background

- Autosomal Dominant Optic Atrophy (ADOA) is the most common inherited optic nerve disorder, characterized by retinal ganglion cell (RGC) loss
- Disease affects 1 in 30,000 people, and causes progressive vision loss; typically presents in the first decade of life
- No therapeutic options are available for ADOA
- 65-90% cases caused by mutations in *OPA1,* a mitochondrial GTPase critical for maintenance of mitochondria health
- Most *OPA1* mutations lead to haploinsufficiency and ~50% of the normal amount of OPA1 protein
- Human stem-cell derived neuronal models of *OPA1* deficiency have differentiation and viability defects[1-4]

TANGO treatment of *OPA1* haploinsufficiency



TANGO ASO promotes NMD exon exclusion, resulting in increased functional mRNA and protein; mutation independent mechanism

Purpose

To generate an induced pluripotent stem cell (iPSCs) model of ADOA to test TANGO ASOs on *OPA1* expression in differentiated RGCs

Methods

Generation of *OPA1* haploinsufficient iPSCs and differentiation of retinal neurospheres

- Guide RNAs designed to *OPA1* exon 9 (NM_015560.2). A normal iPSC line (Gibco) was transfected with gRNA and Cas9 nuclease and stable pools were established. Clonal lines were generated and sequenced to identify targeted mutations, confirmed to have normal karyotype (Karyostat™) and pluripotency (Pluritest™). To generate RGCs, iPSCs were differentiated into retinal neurospheres using a protocol developed in Meyer's lab[5].

***OPA1* gene expression and protein measurement**

- Total *OPA1* mRNA assessed by Taqman qPCR (ddCT method) and protein levels by Western blot. Custom qPCR probes used for detection of NMD exon inclusion. Relative NMD exon inclusion calculated: 2^-dCT(NMD exon-RPL32)/2^-dCT(Total-RPL32) and normalized to wild-type (WT).

Immunofluorescence, flow cytometry, single cell RNAseq

- Neurospheres were dissociated and cultured 5 days in neuron media on PLO/laminin plates, fixed and stained with antibodies and imaged (Image Xpress). For flow cytometry, fresh cell suspensions were incubated with indicated dyes and antibody and analyzed (Guava Incyte, 10,000 gated events). For single cell RNAseq, whole transcriptome gene expression libraries were prepared (10X Genomics 3' Single Cell) on fresh cell suspensions. Data processed with 10x Cell Ranger analysis pipelines and low-quality cells filtered out. Gene counts normalized to cell size factor, per Cell Ranger methods.

ASO treatment of retinal neurospheres

- ASOs added to culture media (days 30-37). Neurospheres were further cultured in ASO-free media until harvest on day 45. For measurement of non-productive *OPA1* mRNA, neurospheres were treated with cycloheximide before whole neurosphere RNA isolation.

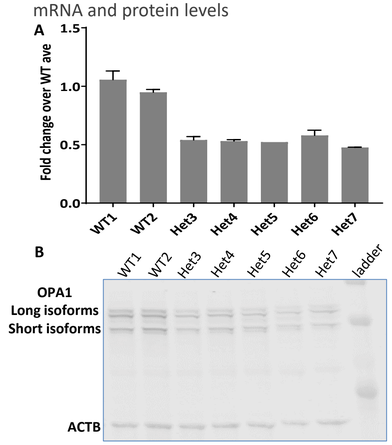
Generation of *OPA1* mutant and isogenic WT iPSCs

Table 1. *OPA1* haploinsufficient iPSCs and isogenic WT controls

Clone	Allele 1	Allele 2	Cell line type	Karyotype	Pluripotency
WT1	WT	WT	WT	Normal	Normal
WT2	WT	WT	WT	Normal	Normal
Het3	WT	1 bp insertion	Heterozygous knock-out	Normal	Normal
Het4	WT	16 bp deletion	Heterozygous knock-out	Normal	Normal
Het5	WT	2 bp insertion	Heterozygous knock-out	Normal	Normal
Het6	WT	2 bp deletion	Heterozygous knock-out	Normal	Normal
Het7	WT	8 bp deletion	Heterozygous knock-out	Normal	Normal

Insertions and deletions introduced by CRISPR/Cas9 into *OPA1* GTPase domain coding sequence

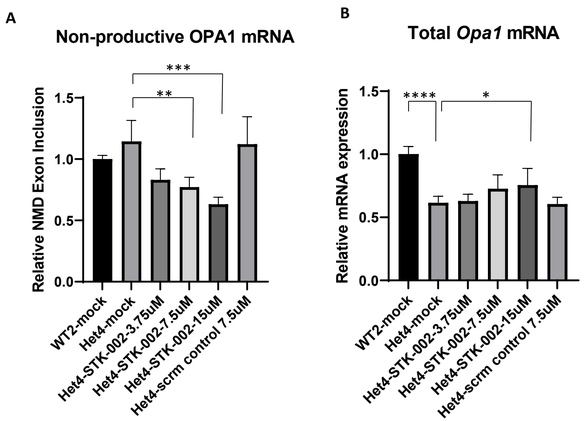
Figure 1. Heterozygous mutant *OPA1* iPSCs have reduced *OPA1* mRNA and protein levels



~50% reduction in total *OPA1* mRNA in mutant lines (Het3-Het7) compared to WT lines (n=2 biological replicates) (A). Reductions in OPA1 protein in *OPA1* mutant lines compared to WT lines (B).

STK-002 reduces *OPA1* NMD exon and increases total mRNA

Figure 7. Targeting of *OPA1* with STK-002 in neurospheres

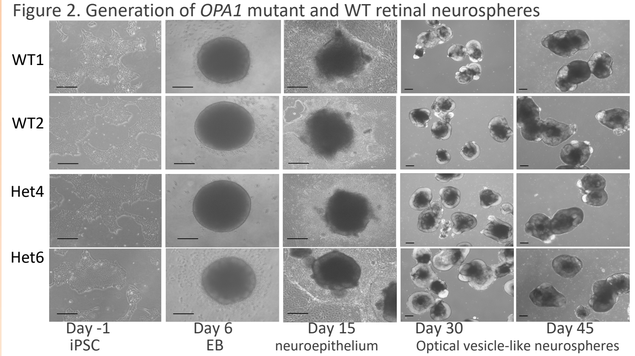


Dose responsive reduction in non-productive *OPA1* mRNA (A) and concomitant increase in total *OPA1* mRNA (B). Averages from 3-6 replicates (3 neurospheres/rep) in 2 separate differentiation experiments and normalized to WT2-mock treated. Scramble control shows no reduction of NMD exon inclusion and no increase in total *OPA1* mRNA.

Significant reductions/increases are observed with higher doses of STK-002. One-way ANOVA, * P<0.05, ** P<0.005, *** P<0.0005, **** P<0.0001.

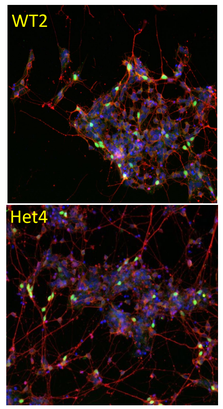
OPA1 mutant iPSC-derived RGCs

Figure 2. Generation of *OPA1* mutant and WT retinal neurospheres



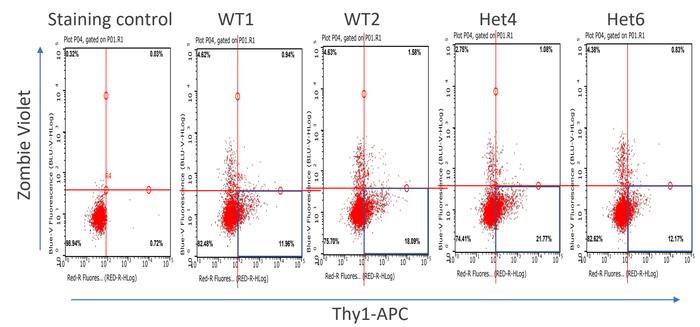
Differentiation of optical vesicle-containing neurospheres. Most neurospheres (WT and Het) display the characteristic bright outer ring of retinal neurospheres (day 30, day 45). No obvious viability or morphological differences between WT and Het. Scale bar 250μm

Figure 3. RGCs in *OPA1* mutant and WT retinal neurospheres



Plated D48 neurospheres. RGCs are BRN3A and TUBB3 positive.

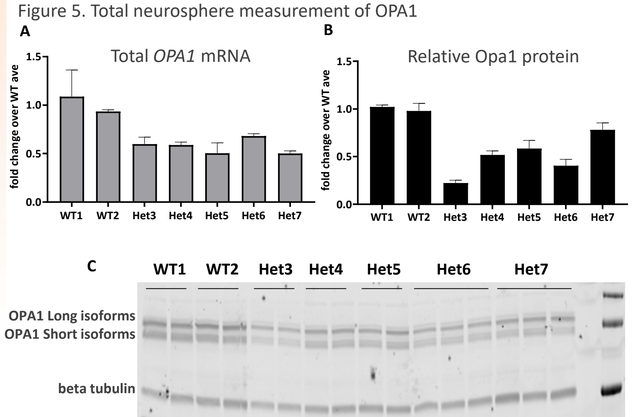
Figure 4. *OPA1* mutant and WT RGCs exhibit high viability



Day 45 neurospheres. Zombie Violet marks dead cells, Thy1-APC marks RGCs. Blue box = live RGCs

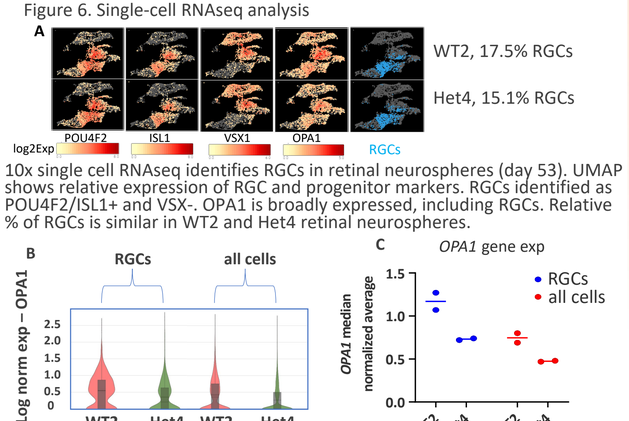
OPA1 expression in retinal neurospheres, RGCs

Figure 5. Total neurosphere measurement of OPA1



OPA1 mRNA (A) and OPA1 protein (B, C) are reduced in total mutant neurospheres (Het3-Het7) compared to WT (day 45). n=2-3 biological replicates, each 2-3 neurospheres.

Figure 6. Single-cell RNAseq analysis



10x single cell RNAseq identifies RGCs in retinal neurospheres (day 53). UMAP shows relative expression of RGC and progenitor markers. RGCs identified as POU4F2/ISL1+ and VSX-. OPA1 is broadly expressed, including RGCs. Relative % of RGCs is similar in WT2 and Het4 retinal neurospheres.

~50% reduction in *OPA1* expression in Het4. Violin plot (B) comparing *OPA1* expression in WT2 and Het4 cells (single biological rep) (C) and median *OPA1* average from 2 biological replicates (C). RGC cells ~800, all cells ~5,000.

Conclusions

- Generated CRISPR iPSCs haploinsufficient for *OPA1* and isogenic WT controls with normal karyotype and pluripotency
- Successfully differentiated *OPA1* mutant iPSCs to form RGCs in a floating retinal neurosphere culture system
- Confirmed haploinsufficiency status in total neurospheres and RGCs
- In these neurospheres, *OPA1* mutant RGCs have similar viability as WT
- STK-002 induced a dose responsive reduction of non-productive *OPA1* mRNA and increase in total *OPA1* mRNA in total neurospheres
- Evaluation of differential gene expression in *OPA1* mutant RGCs (single cell RNAseq) ongoing

References

1. Chen et al., Stem Cell Res Ther, 2016
2. Jonikas et al., Ann Neurol, 2018
3. Ianielli et al., Cell Reports, 2018
4. Caglayan et al., iScience, 2020
5. Fligor et al., Methods Cell Bio, 2020

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