CRISPR knock-out of alpha-synuclein in patient-derived pluripotent stem cell model of Parkinson's disease

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Abstract

The accumulation and aggregation alpha-synuclein protein (a-syn) is a critical event in Parkinson's disease (PD) pathophysiology, impairing neuronal function and contributing to dopaminergic neuronal cell death. The pathogenic genomic triplication of the alpha-synuclein (SNCA) gene (chromosomal locus 4q21, size 1.7Mb) in patients results in early onset rapidly progressive parkinsonism with diffuse Lewy body pathology and severe autonomic involvement (1), suggesting a direct link between increased gene expression of wild-type a-syn and disease development. We have previously shown that overexpression of a-syn as it related to the SNCA genomic triplication is linked to increased susceptibility for oxidative stress and impairment of neuronal maturation in patient-derived fibroblasts or induced pluripotent stem cell (iPSC) models (2,3,4).

The goal of this study was to combine iPSC technology with gene editing to establish isogenic cellular tools which express varying wild-type SNCA gene copy numbers. With this set of new cell lines, we are able to address what are the physiological and detrimental effects of varying a-syn levels, thus greatly simplifying the experimental paradigm that arises when overexpressing proteins or downregulating gene expression.

We have generated CRISPR tools to introduce double-strand breaks in the first coding exon of SNCA gene. Human iPSCs from a SNCA triplication carrier were growth adapted to single cell cloning and were transfected with the CRISPR constructs several rounds before genotyping of individual clones. We generated 11 clones with different mutant alleles relating to 4 knock-out (KO), 3KO, 2KO, 1 KO. The resulting iPSCs were karyotypically normal and expressed pluripotency markers. mRNA expression decreased corresponding to the number of functional copies of the SNCA gene. Dopaminergic neurons derived from these isogenic lines are analyzed for viability, differentiation potential and morphological as well as physiological changes to evaluate the effect of different 'gene doses' of alpha-synuclein.

Here, we present a unique in vitro model system to study the impact of a-syn in an isogenic background. This system will be extremely useful for the study of a-syn associated pathways, drug screening, and the pharmacological modulation of a-syn levels in PD pathophysiology.

Materials and methods

iPSC culture and maintenance: iPSCs were cultured on Geltrex with manual passaging every 6-7 days. Essential 8 media was changed daily.

CRISPR reagent design-build, transfection and screening: cells were adapted to single-cell passaging techniques required for efficient gene editing**.** Five gRNAs (reagents) were designed and built to exon 2 of SNCA (fig 1C). HEK293T were transfected with each reagent to assess cutting at the target locus via Cel-1 assay. Transfection with the nucleases in iPSCs was performed 3 times sequentially (over 6 weeks).
Transfected pools were screened for the presence of the indels indicating repair via NHEJ at the cut-sites. Cel-1 assay was performed to assess the level of cutting at the sites after the each round of transfection. The consolidated clones were initially assessed for knockout alleles utilizing droplet digital PCR. The sequence confirmed clones were further expanded to make the final cell banks for future experiment.

PSC midbrain DA neuron differentiation: Differentiation from iPSCs to DA neurons were using PSC midbrain DA differentiation kit (ThermoFisher, A3147701). iPSCs were cultured for 10 days with specification media to generate floor plate progenitors (FPps). FPps were expanded for 10 more days with expansion media to be either cryopreserved or further maturation. The last 15 days of the differentiation process, FPps were developed into functional DA neurons with maturation media.

Immunocytochemistry: Cells were fixed at day10 and day35 in 4% PFA and permeabilized with 0.3% triton X-100 in PBS for 5 minutes (except cells stained with tyrosine hydroxylase (TH, Millipore, ab152) and β-III-Tubulin (TUJ1, Covance, MMS-435P)) antibodies, blocked with 10% goat serum for 1 hour at RT, and incubated with primary antibodies (at day10, with FOXA2 (ThermoFisher, A29515) and NESTIN (Milipore, MAB5326)) for overnight at 4C. Indirect immunofluorescence staining was performed with Alexa fluor 488 and 555 conjugated H+L antibodies. Fluorescent images were captured on an Nikon Eclipse Ti inverted fluorescence microscope and analyzed with ANDOR Zyla software.

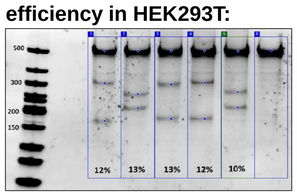
Taqman gene expression analysis: Total RNA was collected using Qiagen Rneasy Minikit from Trizol treated cells at day0, day10, and day35. cDNA was synthetized using the iScript™ cDNA Synthesis Kit. Taqman probes FAM-MGB labeled SNCA, FAM-MGB labeled TH and for normalization VIC-MGB_PL labeled ACTB were used for relative expression analysis. Relative expression levels were calculated with subsequent ΔCT values that were analyzed using CFX software.

sgRNA ID	Target Sequence
CRISPR1	5' GAGAAAACCAAACAGGGTG 3'
CRISPR2	5' GGACTTTCAAAGGCCAAGG 3'
CRISPR3	5' GCTGCTGAGAAAACCAAAC 3'
CRISPR4	5' GCTTCTGCCACACCCTGTT 3'
CRISPR5	5' GCAGCCACAACTCCCTCCT 3'

Figure 1. **A**) SNCA genomic locus of Chr.4q22.1 and CRISPR targeted gene region of the SNCA gene exon 2. **B)** Novel concept for SNCA gene knockout iPSC model. Introduce sequentially guided by CRISPR frame-shift mutations via non-homologous end-joining in the first coding exon of the SNCA gene to generate an 'SNCA gene dosage' model at its endogenous locus. **C)** panel of tested CRISPR guide RNAs used to knock-out SNCA.

Results

Cel-1 assay used to measure reagent (sgRNA) cutting efficiency in HEK293T:



Reagent	Predicted Band Sizes	Cutting %
CRISPR1	305/195 bp	12%
CRISPR2	270/230 bp	13%
CRISPR3	305/195 bp	13%
CRISPR4	305/195 bp	12%
CRISPR5	270/230 bp	10%

Figure 2: HEK293 were transfected with each reagent to assess cutting at the target locus via Cel-1 assay. All five reagents showed cutting efficiency at 10-13%. Lanes 1-5 represent reagents 1 through 5 and lane 6 represents the uncut control.

Cells were transfected with CRISPR3 (highest cutting reagent). In order to increase the chances of getting all four alleles cut, transfection with the nucleases was performed 3 times sequentially over 6 weeks of time period:

Figure 3: Cel-1 assay showing increased cutting efficiency after three consecutive transfections on pooled human iPSCs with SNCA genomic triplication. Lanes 1, 2 and 3 represent cutting with CRISPR3 after each round, with cutting levels reaching 24.5% after the third round. Lanes 4 and 5 represent cutting with another pulse of gRNA 24 hours after the initial transfection. Cutting efficiency reaches 35% with the additional pulse of gRNA. Lane 7 represents the uncut control cells.

SNCA expression in isogenic clones:

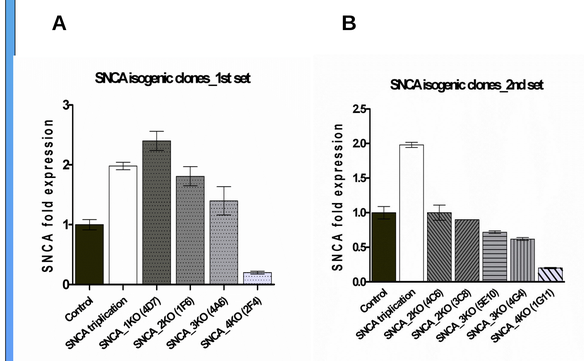


Figure 4: SNCA mRNA expression in iPSCs. CRISPR knock-out (KO) clones are compared to normal control and parental SNCA triplication iPSC clones. A) Isogenic lines show mRNA reduction in proportion to number of KO copies. However, when compared to the parental SNCA triplication, the KO lines express proportionally higher levels of a-syn. There is still residual expression in the SNCA 4KO. B) two other 2KO clones shows similar SNCA expression as control. To note, cell pellets for clones were collected to extract RNA at separate set.

Differentiation of SNCA isogenic iPSC clones into dopaminergic (DA) neurons to investigate phenotypic differences among lines:

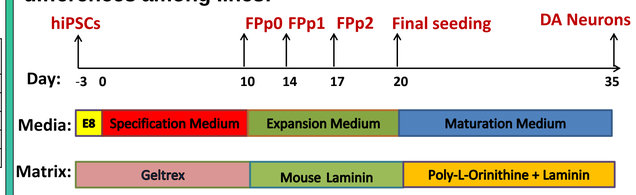


Figure 5: DA differentiation timeline and scheme

Neuronal differentiation protocol exhibits homogenous population of floorplate marker forkhead box A2 (FOXA2):

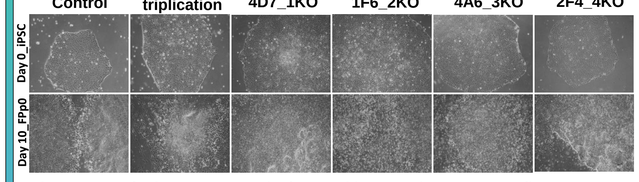


Figure 6: Pluripotent morphology of iPSCs (upper panel). Lower panel exhibits morphology of cells after 10 days treated with specification media.

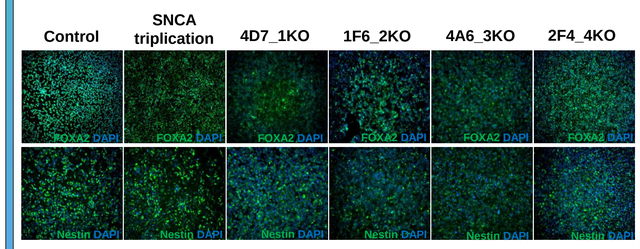


Figure 7: FPp0 at 24 hours after passaging from day10. Cells uniformly express midbrain marker FOXA2 and neuro-precursor marker, NESTIN. Counterstained with DAPI at 10X magnification.

Expression of tyrosine hydroxylase (TH) in DA neurons after 35 days of differentiation:

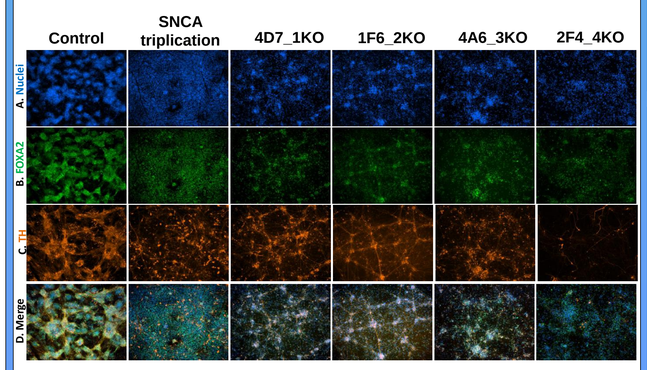


Figure 8: Day35 mature DA neurons stained with FOXA2 and TH. B) DA neurons are still highly positive FOXA2. D) Co-localization of FOXA2 and TH confirms FOXA2 expression is critical to develop DA neurons. In 2F4_4KO line, even though high expression of FOXA2 is noticed (B), but TH expression is very limited (C). Cells were counterstained with DAPI at 10X magnification.

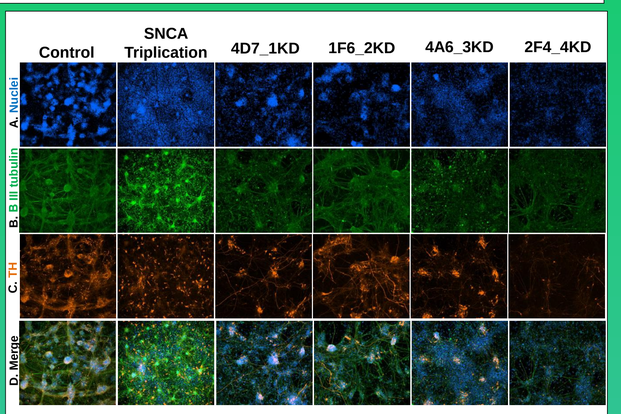


Figure 9: Mature day35 DA neurons stained with TUJ1 for neurons (B) over total cell number (A). C) Cells were also double stained with TH for DA neurons. D) TH positive cells were co-localized with TUJ1 stained cells. Higher density of DA neurons are observed in control, compared to triplication and all isogenic lines. However, among all isogenic lines, DA neurons of 2KO line view morphological and quantity similarity with control. Cells were counterstained with DAPI at 10X magnification.

SNCA and TH expression analysis for different time-points during neuronal differentiation:

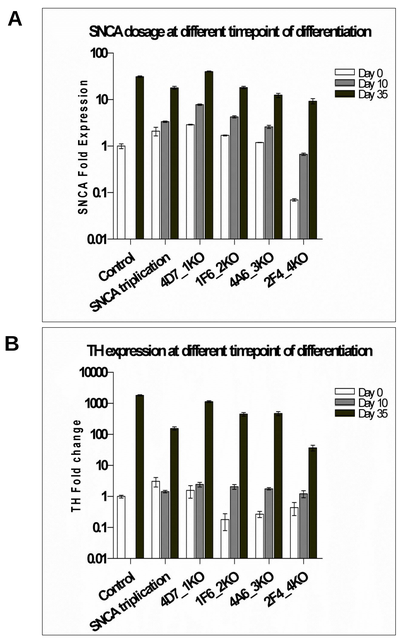


Figure 10: SNCA and TH mRNA expression at day0 (iPSCs), day10 (FPp0) and day35 (mature neurons) determined by Taqman qPCR. A) SNCA expression is almost 60-70 fold higher at day35 compared to day0 or day10. B) TH expression is 100 to 2000 fold higher compared to day0 and day10. At day 35, higher TH noted in 1KO, 2KO, and 3KO lines compared to SNCA triplication line.

Conclusion

- We successfully derived isogenic clones with different KO copies of the SNCA gene and show reduction in a-syn expression in isogenic clones according to wild-type copy number.
- iPSC growth and maintenance was comparable in all studied clones, even the 4KO line did not overtly impact viability and survival of iPSC cultures.
- Neuronal differentiation of 4KO clone showed considerable deficits in neuronal differentiation.

Overall, the sequential targeting of the SNCA locus is an innovative approach to study overexpression and reduced expression of a-syn. This is critical as a-syn downregulation is considered a therapeutic strategy in PD.

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