Genome Editing of α-Synuclein in iPSCs from a Donor with Multiple System Atrophy

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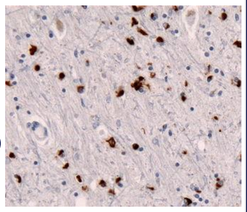
ABSTRACT

Multiple system atrophy (MSA) is a neurodegenerative disorder of primarily glial origin. Clinically it is distinct from Parkinson's disease with predominantly autonomic failure and motor impairment. Pathologically, the principal cellular targets are oligodendrocytes that show abundant glial cytoplasmic inclusion bodies consisting of α-synuclein (SNCA) aggregates and nigrostriatal degeneration. Protein misfolding and aggregation of alpha-synuclein is a common feature across synucleinopathies and an attractive target for drug development.

In order to study the contribution of α-synuclein to the disease phenotype of MSA, we generated induced pluripotent stem cells (iPSCs) from a donor with MSA and then created isogenic lines with SNCA deletions using the GeneArt® Precision TAL-nuclease fusion technology. We designed two sets of TALEs to create deletions in exon 2 of the SNCA gene in patient derived iPSCs via non-homologous end-joining (NHEJ). After functional verification of the SNCA TALENs carried out in HEK and U-2 OS cells, and confirmed with the Surveyor assay and Sanger sequencing, iPSCs from the MSA donor were edited with SNCA TALENs. Colonies that showed positive cuts in the Surveyor assay were picked and expanded. These colonies were further characterized by next gen-sequencing on the Ion Torrent PGM instrument to identify clonal populations with SNCA deletional mutations. The edited clones with SNCA heterozygous deletion mutation are karyotypically normal and express pluripotency markers. These clones are in the process of re-editing to generate homozygous deletions, after which they will be further differentiated into appropriate cell types for phenotypic studies. In conclusion, TAL technology can be applied to generate isogenic disease iPSCs for studying the contribution of SNCA in the disease phenotype of MSA.

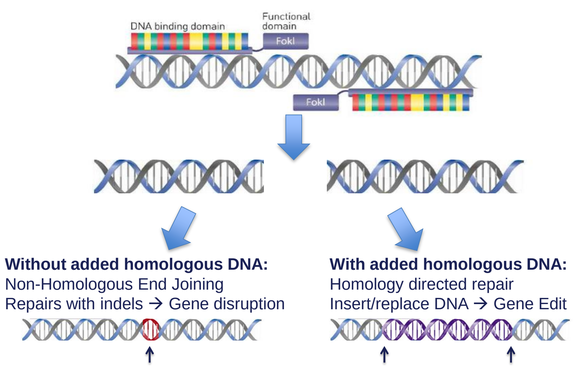
INTRODUCTION

Multiple System Atrophy (MSA)



- Neurodegenerative disorder with Parkinsonian features (tremors at rest, rigidity of muscles, slowness in moving)
- No known genetic basis or risk-factors.
- Poor response to standard dopamine-replacement therapy (levadopa) used in treating PD
- Characterized by glial α-synuclein deposits (Lewy Bodies)

Transcription Activator Like (TAL) Effector Nuclease



Without added homologous DNA:
Non-Homologous End Joining
Repairs with indels → Gene disruption

With added homologous DNA:
Homology directed repair
Insert/replace DNA → Gene Edit

MATERIALS AND METHODS

Cell culture

All cell culture media, components, growth factors and pluripotency characterization antibodies are from Life Technologies. iPSCs are cultured on irradiated MEF dishes in KSR hiPS medium (KnockOut™ DMEM/F-12, KnockOut™ Serum Replacement, MEM Non-Essential Amino Acids, GlutaMAX™ Supplement, 2-Mercaptoethanol and FGF-Basic (AA1-155) Recombinant Human Protein-10 ng/mL final). iPSCs in feeder free conditions were grown on Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix in StemPro® hESC SFM medium (DMEM\F-12+GlutaMAX™, StemPro® hESC supplement, Bovine Serum Albumin and FGF-Basic (AA1-155) Recombinant Human Protein-20 ng/mL final).

SNCA TAL Expression Constructs and Transfection/Electroporation

SNCA TAL pair were cloned into an EF1α expression vector. For transfection into U-2 OS cells, 0.5 million cells were plated onto each well of a 6-well plate and transfected with 2.5 ug of each TALE expression plasmid with Lipofectamine LTX (Life Technologies) according to the manufacture's protocol. 72 hours after transfection, cells were harvested for genomic DNA isolation. MSA iPSCs grown on feeder-free Geltrex™ coated plates in StemPro hiPS medium were disassociated into single cells with Accutase (Life Technologies). About 0.8X10^6 cells were electroporated with 2.5 ug each of SNCA forward and SNCA reverse TALE expression constructs using the Neon™ system (system settings: 1400V, 10ms, 2 pulses). After electroporation, cells were plated onto MEF culture dishes (20,000 cells/cm^2) in MEF conditioned KSR hiPS medium supplemented with 10 ng/mL bFGF.

Surveyor® Assay and TA cloning/Sanger Sequencing

Surveyor® Assay was performed using a kit from Transgenomic. Genomic DNA was prepared from recovered iPSC colonies, followed by a nested PCR reaction over the SNCA region to be edited. The PCR product (approx. 400bp) is denatured and re-annealed. If editing occurred, heteroduplexes are formed. Surveyor enzyme recognizes the mismatch and cleaves the PCR fragment. Positive results from the surveyor assay were confirmed by cloning the 400bp PCR product into Zero Blunt® TOPO® PCR Cloning Kit and sanger sequencing of the resulting colonies using BigDye® Terminator v3.1 Cycle Sequencing Kit.

Using the Ion Personal Genome Machine® (PGM™) for Detecting Gene Editing

We developed a novel method to rapidly determine sequence changes detected by Surveyor® assay and to determine the frequency of the modifications in the cell population for multiple samples. The region of interest is amplified using starting material already generated from the surveyor assay. Amplicons from multiple samples can be barcoded and sequenced using the Ion Personal Genome Machine (PGM™). The resulting sequence yields thousands of reads per sample which allows the determination of not only sequence modification but frequency of modifications in the cell population.

PCR I material from Surveyor® assay used as starting material for PGM™ sequencing – PCR II rxn performed using primers for 200bp fragment

⬇

200bp fragment used to construct library using Ion Plus Fragment Library Kit and Ion Xpress™ Bar Code Adapters Kit for multiple samples then quantitated using Ion Library Quantitation Kit

⬇

Library templated using Ion One Touch™ 200 Template Kit V2 DL

⬇

Templated library sequenced using Ion PGM™ 200 Sequencing Kit

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Sequence analyzed using IGV 2.2 software

RESULTS

Figure 1. SNCA GeneArt® Precision TAL Design and Verification

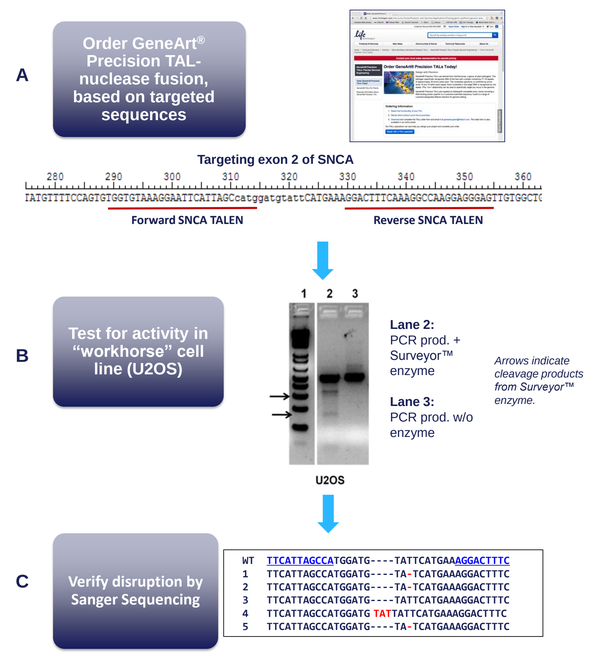
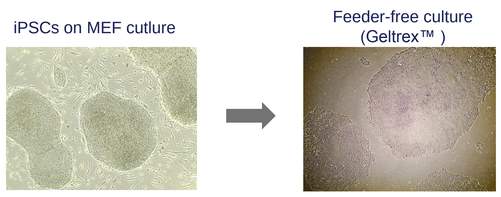
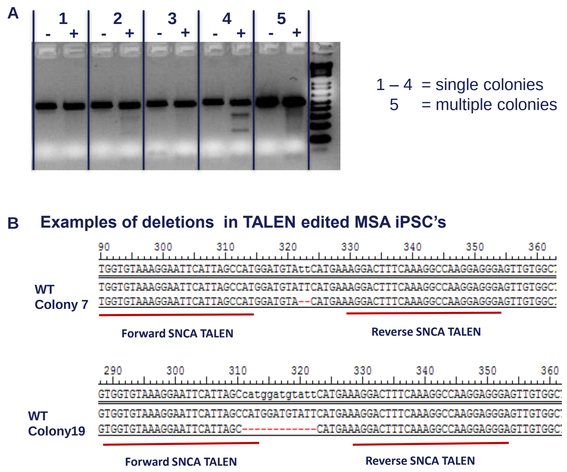


Figure 2. Adaptation of MSA iPSCs to Feeder-free Culture

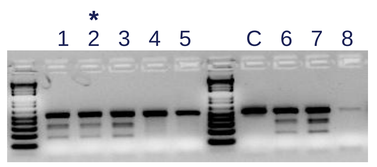


In preparation for electroporation of iPSCs with TAL expression vectors the cells were adapted to a feeder free system. Cells growing on irradiated MEFs were passaged using an EZ passage tool into dishes coated with Geltrex and cultured in conditioned KSR hiPS media (KSR hiPS media incubated with MEF feeder layer for 24 hrs then human basic FGF added and filtered). After two days of culture in conditioned hiPSC media, StemPro® media was added in a stepwise fashion starting with 25% StemPro® and 75% conditioned hiPSC media. This process was continued by increasing the amount of StemPro® media by 25% every two days until the cells were in 100% StemPro® media.

Figure 3. SNCA Editing in MSA iPSCs

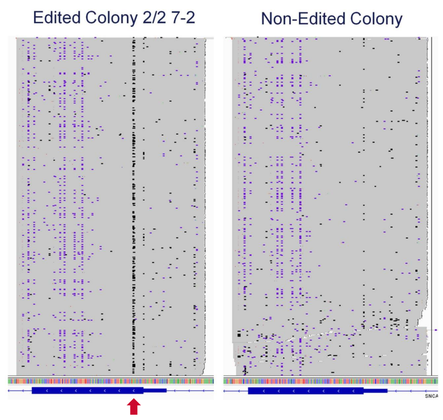


C Colony 7 expansion



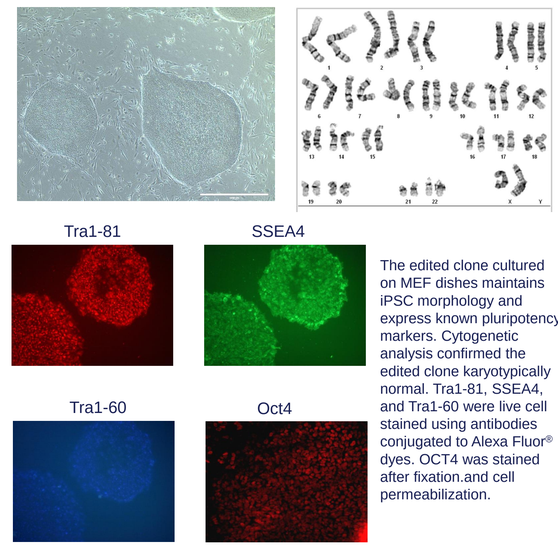
Surveyor® assay positive colonies. * Deletion confirmed by sequence

D Ion Personal Genome Machine® (PGM™) for Detecting Gene Editing



iPS cells transfected with TAL expression plasmids were plated onto a MEF feeder layer by limiting dilution and cultured until colonies were large enough to be tested by Surveyor® assay for editing. **A.** Representative Surveyor® assay results showing the degradation of heteroduplexes in the presence of Surveyor® enzyme. **B.** Amplicons from Surveyor® positive colonies were TOPO® cloned and sequenced using sanger methodology to confirm editing at the SNCA locus. **C.** To obtain a clonal population of iPS cells with the TAL induced mutation the cells were dissociated with StemPro® Accutase® and replated on MEF feeder layers using limited dilution. Surveyor® assay was used to identify candidates for a clonal population. **D.** PGM™ sequencing of clonal candidates confirms that a clonal population containing a heterozygous mutation in the SNCA locus was achieved.

Figure 4. Characterization of the Edited Clone



The edited clone cultured on MEF dishes maintains iPSC morphology and express known pluripotency markers. Cytogenetic analysis confirmed the edited clone karyotypically normal. Tra1-81, SSEA4, and Tra1-60 were live cell stained using antibodies conjugated to Alexa Fluor® dyes. OCT4 was stained after fixation.and cell permeabilization.

SUMMARY

We generated an isogenic MSA iPSC clone with a heterozygous deletion of α-synuclein using the GeneArt® Precision TAL technology.

➢ 15% of the recovered colonies were positive in Surveyor® Assay, i.e. NHEJ mutation occurred.

➢We developed and optimized PGM sequencing method that allows for rapidly detecting sequence changes in Surveyor® Assay positive colonies and for determining homozygous or heterozygous editing.

➢ The resulting edited iPSCs express the expected pluripotency markers and are karyotypically normal.

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TRADEMARKS/LICENSING

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