Gene engineering of the alpha-synuclein gene by CRISPR/Cas9 in Parkinson's disease patient-derived stem cell cultures

Allen Y. Kim[1,2] and Birgitt Schüle[2]

[1] *Department of Biological Sciences, San José State University, San Jose, CA 95192;*
[2] *Basic Research, Parkinson's Institute and Clinical Center, Sunnyvale, CA 94085*

Abstract

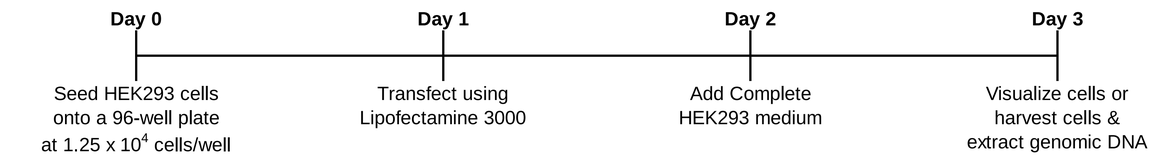
The overall goal of the study is to better understand the role of the alpha-synuclein (SNCA) gene in neurodegeneration of Parkinson's disease (PD). PD is a neurodegenerative disease that is most commonly characterized by the accumulation of the pre-synaptic protein alpha-synuclein into intracellular aggregates called Lewy bodies in neurons. Although the biological function of alpha-synuclein is still not fully understood, studies have shown that missense mutations (NM_000345.3 (SNCA) c.88G>C (p.Ala30Pro), c.136G>A (p.Glu46Lys), c.150T>G (p.His50Gln), c.152G>A (p.Gly51Asp), c.157G>A (p.Ala53Thr)) and multiplications (duplication & triplication) of SNCA are linked to familial forms of PD. This suggests that molecular changes of the alpha-synuclein protein and overexpression of wild-type alpha-synuclein protein both promote protein aggregation and eventual neurodegeneration. Therefore, a reduction of alpha-synuclein protein in neurons has been thought of as a rational therapeutic strategy for treating PD. In the past decade, several groups have made attempts at examining the effects of reducing the presence of intracellular alpha-synuclein in vitro and in vivo utilizing two main approaches: 1) targeting SNCA mRNA transcripts via the RNA interference and 2) targeting alpha-synuclein protein itself via antibody immunotherapies. However, these studies have reported conflicting results, and the effects of alpha-synuclein knockdown remain controversial. And thus, there is a great need for additional research to advance our current knowledge of alpha-synuclein.

To better understand the effects of SNCA knockdown in PD pathogenesis, we interrogate the coding region of the SNCA gene. This is an alternative approach of studying the effect of SNCA knockdown compared to prior studies which employed approaches that target mRNA or protein. We are currently utilizing the genome editing system CRISPR/Cas9 to knockdown SNCA in vitro in cell lines derived from a PD patient with an SNCA triplication which has been shown to exhibit PD-related phenotypes in culture. We have designed several CRISPR/Cas9 systems that specifically target SNCA exons 2 through 5 and validated them in HEK293 cells in vitro. In addition to investigating the effects of SNCA knockdown, we are also testing the efficiency of multiplex CRISPR/Cas9 system to target multiple SNCA exons at once. We believe that this approach of knocking down SNCA using CRISPR/Cas9 system will enable reverse genetic studies and will allow the investigation of any potential therapeutic effects associated with SNCA knockdown in patient-derived stem cell cultures in vitro.

Materials & Methods

***Cell Culture*:** Human embryonic kidney 293 (HEK293) cell line was maintained in Dulbecco's high glucose modified Eagle's medium (HyClone) supplemented with 10% fetal bovine serum (HyClone) and 2mM GlutaMAX (Life Technologies) at 37°C with 5% CO_2.

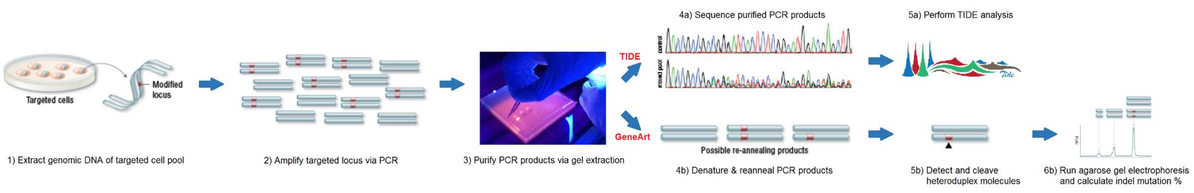
Transfection Optimization: Passage 16 HEK293 cells were seeded onto a 96-well plate (Thermo Scientific) at a density of 1.25 x 10^4 cells/well, 24 hr prior to transfection. Cells were transfected using Lipofectamine 3000 (Life Technologies) at 70-80% confluency following the manufacturer's recommended protocol for transfection optimization. 100, 200, 300, and 400ng of empty GFP-tagged Cas9 DNA plasmid pX458 (Addgene plasmid #48138) were transfected at 1:1, 1:2, and 1:3 DNA:Lipofectamine reagent ratio. Optimal transfection parameters were determined via fluorescence microscopy.



Single Guide RNA Designs and CRISPR/Cas9 Constructs: Three designs of single guide RNA (sgRNA) were generated using the online CRISPR Design Tool (http://crispr.mit.edu/) targeting exon 2 of the *SNCA* gene (hg19/GRCh37; NM_000345.3). sgRNA-subcloned pGS-U6-gRNA (GenScript) and humanized Cas9 vector pGS-CMV-hCas9 (GenScript) were used.

Transfection Parameters for Determination of Optimal Indel Mutation Formation: Passage 17 HEK293 cells were co-transfected with pGS-U6-gRNA and pGS-CMV-hCas9 at equimolar concentrations of 100, 200, 300, and 400ng of total plasmid DNA with 1:2 ratio of DNA:Lipofectamine reagent as described above. Control cells were transfected with only the pGS-CMV-hCas9 plasmid. 100µL of Complete HEK293 medium was added to each well 24 hr post-transfection. Cells were harvested 48 hr post-transfection, and their genomic DNA (gDNA) was extracted using GeneArt Genomic Cleavage Detection Kit (Life Technologies).

Comparison of Methods to analyze cutting efficiency of CRISPR system: TopTaq Master Mix Kit (Qiagen) was used with 0.2µM primers (FWD: CTGGAAAAGCAAACAGTCGCA; REV: AGCCAAGATGGATGGGAGATG; 544bp amplicon) to amplify *SNCA* exon 2 region of the extracted gDNA. PCR was performed following the manufacturer's recommended protocol at 40 cycles and at an annealing temperature of 65°C. PCR products were ran on 2% NuSieve GTG Agarose (Lonza) gels and purified using QIAquick Gel Extraction Kit (Qiagen). Concentrations of purified PCR products were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies). Subsequently, two analyses were carried out to assess indel mutation formation: 1) Bioinformatics – Tracking of Indels by DEcomposition (TIDE) (*Brinkman et al, Nucl. Acids Res. (2014)*); and 2) Enzymatic – GeneArt Genomic Cleavage Detection Kit.



Tracking of Indels by Decompositions (TIDE) Analysis is a web tool that requires two standard capillary sequencing files to quantify genome editing efficiency in the DNA of a targeted cell pool using a decomposition algorithm. Standard capillary sequencing reactions were outsourced and performed on the purified PCR products using ABI 3730xl DNA Analyzer platform (Sequetech Corporation). Sequencing results were then analyzed using the TIDE website (http://tide.nki.nl/) in default parameter. Indel mutation % values obtained from duplicates were averaged.

GeneArt Genomic Cleavage Detection Kit contains an enzyme that detects and cleaves heteroduplex double stranded DNA molecules. Digested DNA fragments are then analyzed via agarose gel electrophoresis and band densitometry, producing semi-quantitative indel mutation % data. 100ng of purified PCR products (duplicates were pooled in equal amounts) were digested with Detection Enzyme following the manufacturer's recommended protocol. Samples were analyzed via agarose gel electrophoresis on 2% E-Gel EX Agarose Gels (Life Technologies). Band intensities were quantified using the image processing software ImageJ (National Institute of Health), and indel formation % was calculated using the following formula: $Indel\ \% = 100 \times (1 - \sqrt{(1 - f_{cut})}$ where f_{cut} represents the fraction of cleaved PCR products (*Ran et al, Nat Protoc. (2013)*).

Results

Optimization of Lipofectamine 3000 Transfection via Fluorescence Microscopy

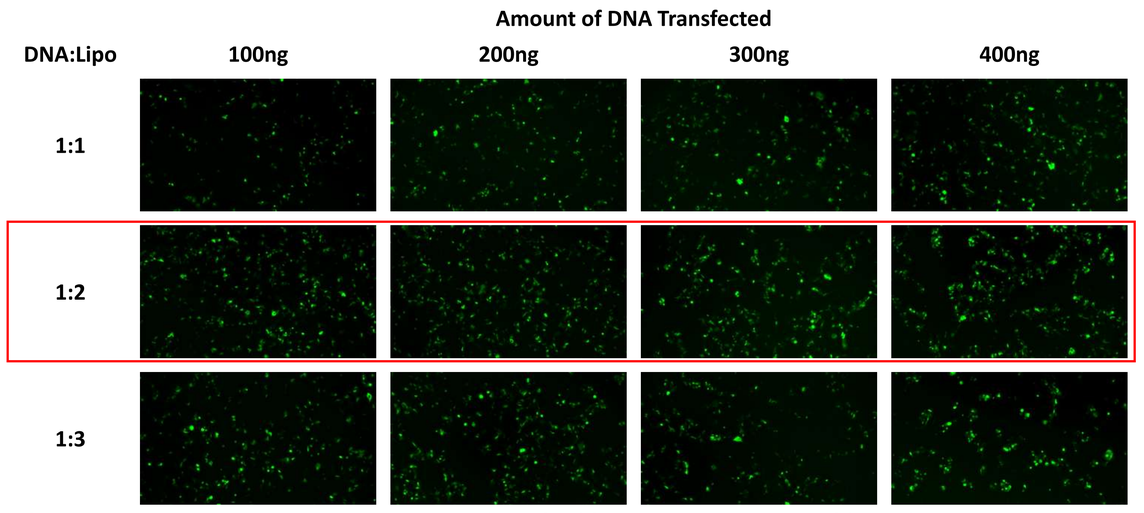


Figure 1. Passage 16 HEK293 cells transfected with empty pX458 plasmid, 48 hr post-transfection. Qualitatively, the optimal DNA:Lipofectamine reagent ratio was determined to be 1:2, having the highest GFP expression in every DNA condition. Reduction in cell viability was observed in 4 conditions: 1:2 & 1:3 ratios for 300ng & 400ng of DNA. Cells were observed under fluorescence microscopy at 100X.

Comparison of Indel Mutation Detection Methods

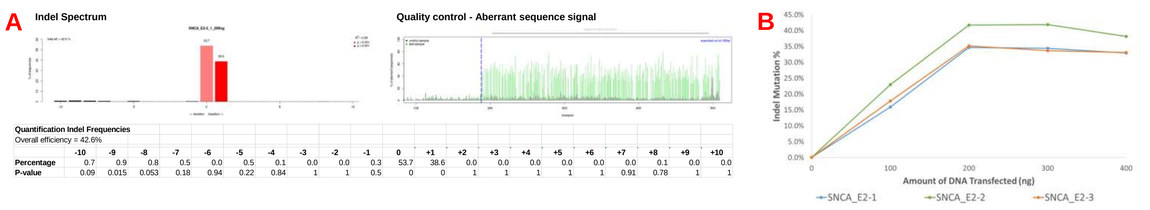


Figure 2. Sequencing and TIDE Analysis results for P17 HEK293 cells transfected with pGS-U6-gRNA and pGS-CMV-hCas9 plasmids. (A) Results of TIDE Analysis for the sample transfected with 200ng of SNCA_E2-2 design are shown and serve as a representation. (B) Plot of average indel mutation % at varying DNA amounts (n = 2).

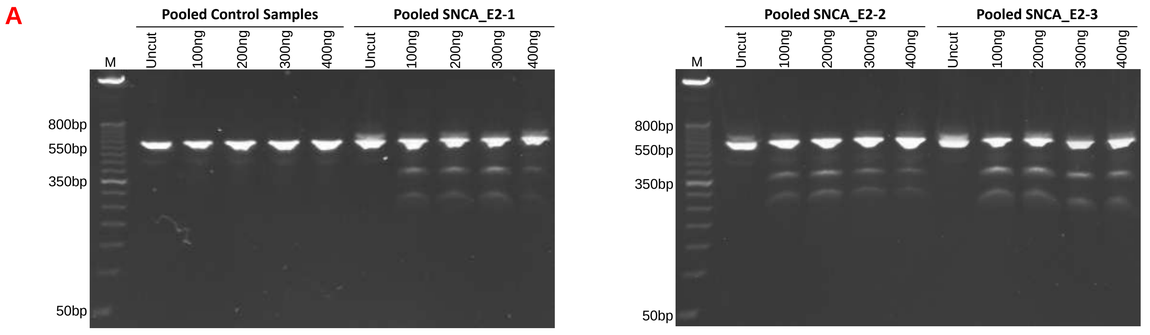


Figure 3. GeneArt Genomic Cleavage Detection Kit results for P17 HEK293 cells transfected with pGS-U6-gRNA and pGS-CMV-hCas9 plasmids. (A) Gel electrophoresis of Detection Enzyme-digested DNA fragments. The theoretical size for parent band is 544bp, while ~334bp & ~210bp daughter bands are expected. Duplicates were pooled together at equal amounts and 100ng of DNA products were digested. (B) Comparison of indel mutation % values from TIDE Analysis and GeneArt Genomic Cleavage Kit.

B

Amount of DNA Transfected	SNCA_E2-1		SNCA_E2-2		SNCA_E2-3	
	TIDE	*GeneArt*	*TIDE*	*GeneArt*	*TIDE*	*GeneArt*
0ng	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
100ng	15.9%	6.6%	23.0%	8.0%	17.8%	14.9%
200ng	34.6%	6.1%	41.7%	12.1%	35.1%	13.6%
300ng	34.5%	9.7%	41.9%	7.1%	33.7%	12.1%
400ng	33.0%	1.7%	38.2%	3.1%	33.1%	9.2%

Challenges & Conclusions

- Indel mutation % values can differ greatly depending on the detection method. Therefore, it is critical for researchers to understand the advantages and limitations of each method and make a rational decision on the appropriate detection method for their research.
- There is a need for a new detection method that is more reliable, precise, and reproducible.
- There are many variables to consider when conducting research utilizing a genome engineering system such as CRISPR/Cas9. Therefore, standards need to be established in order to improve reproducibility of scientific research and to facilitate scientific innovation in the genome editing community.

Acknowledgements

- We acknowledge members of the Birgitt Schüle Lab and Jeremy Nichols Lab for their scientific expertise & support.
- This work is supported by the California Institute for Regenerative Medicine (Grant #TB1-01195).
- pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138).