Advanced Gene-Targeting Methods to Generate Cell Line Models that Preserve Native Regulatory Elements for Efficient High-Throughput Drug Screenings

Huynh P. Duong, Warunee Dansithong, Daniel R. Scoles, and Stefan M. Pulst

Abstract

Many genes were known to cause Parkinsonism. One of these genes is the *SNCA* which codes for α-synuclein. Elevated levels of misfolded α-synuclein cause dopaminergic neuronal loss in PD. Misfolded α-synuclein clearance and HADC inhibitors treatment were neuroprotective to dopaminergic neurons in PD animal and cell models suggesting that manipulating transcriptional gene expression can be used to prevent dopaminergic neuronal death in PD patients. However, there is no cell line model that has the entire *SNCA* regulatory elements for identifying compounds that act on the *SNCA* regulatory elements. We aimed to produce cell lines that express either α-synuclein-luciferase or α-synuclein-GFP for high-throughput drug screenings for compounds that regulate *SNCA* transcriptional functions. We constructed a pair of ZFN-FokI and donor plasmids consisting of a GFP or Luciferase gene flanked by ~800 bp sequences up- and downstream of the ZFN-FokI cleaved site of the *SNCA* gene. The ZFN-FokI and donor plasmids were cotransfected into growing SH-SY5Y cells, selected by 10 μg/ml puromycin, and confirmed by RT-PCR, Western blots, and chemical treatments. Two SH-SY5Y cell lines expressing α-synuclein-luciferase (Luc6B) or α-synuclein-GFP (GFP12) were generated. RT-PCR confirmed that the reporter gene was located at the desired site. Western blots using anti-GFP, anti-α-synuclein, and anti-luciferase antibodies confirmed that both cell lines produced the desired α-synuclein fusion proteins. Valproic acid treatments of the Luc6B and GFP12 cell lines significantly increased the expression of levels α-synuclein luciferase and α-synuclein-GFP. In conclusion, we have used the ZFN method successfully to generate cell lines which will be useful in high-throughput drug screenings to identify compounds that can inhibit the elevation or expression of α-synuclein. These cell lines provide unique tools for drug screens as they include human *SNCA* regulatory control regions in promoters, introns, and even distant sites that potentially interact through chromatin loops.

Keywords

Zinc-finger-nuclease (ZFN) • α-synuclein • Parkinson's disease • High throughput compound screening

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1 Introduction

Parkinson's disease 1 (PARK1) is an autosomal dominant disorder caused by mutations and duplication of the *SNCA* gene that encodes α-synuclein [1–4]. Besides missense mutations which occur rarely in familiar PARK1, *SNCA* duplications and triplications have been found in both sporadic PD and PARK1 [1–5]. *SNCA* gene multiplications suggested that gene dosage and overexpression of wildtype α-synuclein are important factors in causing neurodegeneration in α-synucleinopathies [6]. This hypothesis is supported by observations that elevations of α-synuclein levels caused cell death in PD patients [7–9], cellular, and animal models of parkinsonism [10–12].

Based on these observations, it is theorized that reducing the levels of α-synuclein will be beneficial to patients with α-synucleinopathies, including Parkinson's Disease. However compounds reducing the levels of α-synuclein are rare and have not been found beneficial to PD patients. To identify compounds that reduce the levels of α-synuclein, we intended to use high throughput compound screenings (htps) using dopamine producing cell lines specifically created for htps.

This report introduces the generation of cell lines created by in vitro insertion of the luciferase or GFP reporter gene in-frame with the C-terminal amino acid of the α-synuclein gene using the Zinc-Finger-Nuclease (ZFN) method [13].

In this summary, I will describe the ZFN method in detail and briefly introduce the use of the CRISPR-cas9 method to create a specific cleavage site in situ on the genome of the interested gene. Successfully htps need models for the detection of reactive compounds. These models can be bacteria, yeast, animal, or stably expressed cell line models that express the target gene. Cell lines is a good model for identifying compounds that regulate gene expression or protein processing. Before Zinc Finger Nuclease, TALEN, and CRISPR-cas9 methods that creates double-strand DNA breakage at the investigator's desired DNA site, stably expressed cell lines were often created by plasmid based method which consists of a fragment of the targeted gene containing either the gene promoter/enhancer regulator elements or the gene open reading frame in frame with a marker gene such the luciferase cDNA (Fig. 1). However, approaches using expression plasmids may not ideal since plasmid sizes are limited and randomly integrated in the genome. In addition, gene regulator elements may be locate far from transcription start sites making it impossible to capture all regulatory elements. For many genes, the promoter/enhancer/repressor elements can be as far upstream as 8000 bp or farther. One of these genes is the α-synuclein gene, *SNCA*, which has the gene promoter/enhancer located more than 8000 bp from the transcription start site. Therefore, the plasmid based method is not a suitable method to generate cell lines for HTS to isolate compounds that to suppress the expression and/or clearance of α-synuclein. For these reasons, we used the Zinc Finger Nuclease (ZFN) method to modify the *SNCA* gene at the genomic level.

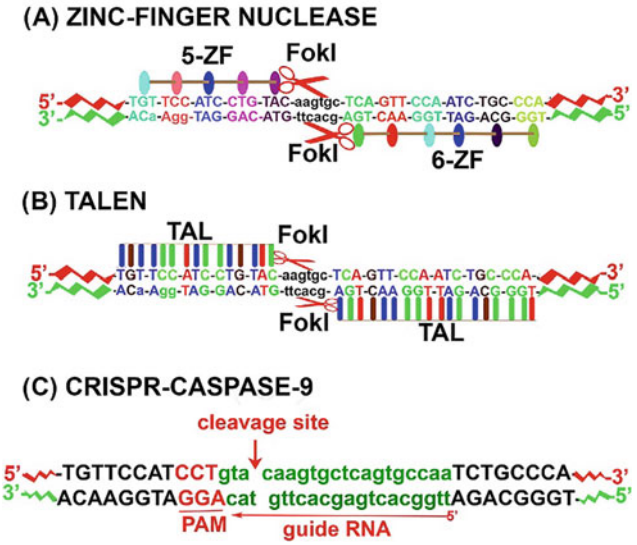

Fig. 1 Genomic DNA Editing Methods. Currently, there are three common genomic DNA editing methods that utilized the composition of the nucleotide sequences of a gene for specific genomic DNA editing. These include the *ZFN* (Zinc Finger Nuclease), *TALEN* (transcription activator-like effector nucleases), and *CRISPR*-Caspase9 (cluster regularly interspaced short palindromic repeat)

2 Methods

Currently, there are three genomic DNA editing methods available for scientists to modify their favorite genes in situ (Fig. 1). Briefly, the Zinc Finger Nuclease (ZFN) and the Transcription Activator-like Effector Nuclease (TALEN) are artificial restriction enzymes that are engineered to bind to any desired short nucleotide sequence of a gene and create a specific DNA breakage there. The ZFN [13] consists of a zinc-finger containing eukaryotic transcription factor linked with a DNA cleaving enzyme FokI. Each ZF recognizes 3 pairs of nucleotides, and ZFN normally contains 3–6 zinc fingers for efficient DNA binding. TALEN [14], on the other hand, consists of a transcription activator-like effector (TALE) and a nuclease such as FokI. The TAL effector is synthesized by *Xanthomonas* bacteria. The TAL effector DNA binding domain possesses a conserved highly repeated 33–34 amino acid residues with different repeat variable residues (RVD) at the 12th and 13th positions. TAL effector DNA binding domains with different RVD recognizes specific nucleotide base pairs. Both ZFN and TALEN create double strand breakage (DSB) which can be repaired by

nonhomologous or homologous repair. One of the disadvantages of both the ZFN and TALEN is that zinc fingers or the transcription-like effectors have to be engineered based on the sequences immediately upstream and downstream of the target site.

In contrast, the CRISPR-Cas9 [15] uses a synthetic guide RNA (sgRNA) upstream of a PAM site (5′**NGG**3′) linked to caspase 3 with cuts 3 bp upstream of the PAM site. This method is simpler than the ZFN and TALEN methods as the sgRNA is easier to select, synthesize, and clone into a CRISPR-Cas9 plasmid system. There are online resources to assist in the design of sgRNA, one such site is the http://crispr.mit.edu/ homepage.

All three methods, ZFN, TALEN, and CRISPR-Cas9 create in situ double strand DNA breakage that can be repaired by either nonhomologus end joining (NHEJ or homologous repair by donor plasmids containing flanking templa t de erived from the interested gene.

In this paper, we will describe the use of the ZFN method to introduce a reporter cDNA, luciferase or GFP, in-frame with the last codon of the alpha synuclein gene *SNCA*. Figure 2 schematically describes the strategy of the ZFN method.

Construction and Verification of ZFNs: A pair of ZFN-FokI plasmids was custom made by Sigma Aldrich using their library of Zinc Finger transcription factors. To create a double strand DNA breakage (DSB), two ZFN plasmids were generated to express ZF-FokI restriction enzymes that bind to the (+) and (−) strands at the designated site and create a DSB at the target site. To verify the specificity of the ZFN, ZFN plasmids were transfected to HEK293 cells and DNA and RNA were isolated. PCR were conducted using PCR primers flanking the predicted breakage point which generated an uncut PCR product of 329 bp. The PCR product 329 bp fragment was then treated with Cel-1 nuclease, which cleaved the mismatched short oligonucleotide resulting from random repairing of the DSB, cut the 329 bp fragment into two fragments of 195 and 134 bp [16]. Upon sequencing of the cleaved products, the cleavage site was identified to be at the AAGTGC sequence located 59 bp downstream of the stop codon of the alpha synuclein gene. Although the cleavage site is located 59 bp downstream of the target site, specific introduction of the marker cDNA was achieved since specific DNA homologous repair can be efficiently achieved within 100 bp of a designated target site. The donor plasmid consists of (1) the right and left arms which are sequences upstream and downstream of the target site; (2) the modified DNA fragment (mutated gene) or foreign gene you desired to insert into the gene (Fig. 2). For the *SNCA* donor plasmid, the

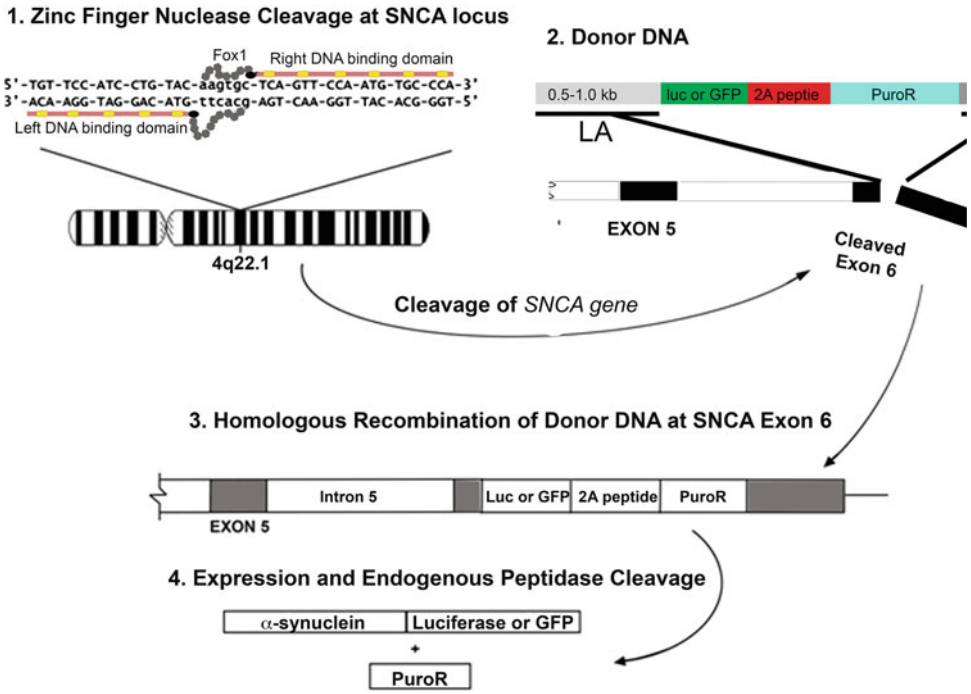

Fig. 2 Schematic diagrams of the upstream and downstream *SNCA* Zinc Finger Nuclease, the donor plasmid with *Left* and *Right* Arms with Luciferase/GFP cDNA and puromycin resistant cDNAs. After the ZFN plasmids are cotransfected the donor plasmid into cells, the ZFN creates double-strand breakage (DSB), and the donor plasmid will serve as template for homologous repair. Once repaired, the modified *SNCA* gene will include *SNCA*-luc (or GFP)-2A-puroR and express a modified α-synuclein-luc (or GFP)-2A-puroR mRNA. This modified mRNA will generate a large α-synuclein fused to the luc (or GFP), 2A peptide, and the puromycin resistance protein. The puromycin resistance protein is then cleaved from the α-synuclein-luc (or GFP) fusion protein by endopeptidase. Figure was adapted from Dansithong et al. 2015 [16]

foreign gene consists of the luciferase or GFP cDNA connected to *Puro* cDNA encoding puromycin resistance via an oligonucleotide encoding for a 2A peptide signal. The 2A peptide signal allows for specific endogenous protease cleavage of puromycin from the α-synuclein-luciferase or α-synuclein-GFP fusion protein. Factors important for making an efficient donor plasmid include: (1) the modified site should not be farther than 100 base pairs (bp) from the double-strand breakage (DSB), (2) the right and left arms should not be less than 500 bp or more than 1000 bp to serve as efficient templates for repairs.

For the *SNCA* gene, two donor plasmids were constructed (Fig. 2). Each donor plasmid contains two flanking *SNCA* fragments immediately upstream and downstream of the *SNCA* mRNAstop codon. The upstream flanking fragment is fused to a reporter cDNA (GFP or luciferase cDNA). The GFP-A2-Puro or luciferase-A2-Puro gene cassette is flanked by ~800 bp sequences up- and downstream of the ZFN-FokI DSB site of the *SNCA* gene. Therefore, the mature modified *SNCA* mRNA will contain the α-synuclein mRNA-fused in-frame with either the GFP or luciferase coding sequence connected to the puromycin resistance gene by the 2A oligonucleotide (α-synuclein-luciferase or GFP-2A-Puro mRNA). Translation of the modified α-synuclein mRNA generates a large protein α-synuclein–luc-2A-puromycin, and host endopeptidases will cleave the 2A peptide out separating α-synuclein-luciferase (or GFP) fusion protein from puromycin for antibiotic selection.

3 Results

To generate a stable cell line expressing a α-synuclein-luciferase or α-synuclein-GFP. The upstream and downstream ZFN-FokI plasmids were cotransfected with the donor plasmid into SH-SY5Y cells by lipofectamine, and cell lines were selected by 1–4 μg/ml puromycin treatment for 1–2 weeks. To confirm specific gene expression of the cell lines, RNA and protein extracts were analyzed by RT-PCR, qPCR, and Western blots. Additional confirmatory experiments may include treatment of the cell lines with chemicals or siRNA that affect the expression of the modified gene such as *SNCA* siRNA or valproic acid treatments for α-synuclein expression, as described further in the next paragraph (Fig. 3).

We analyzed the stably transfected cell line (Luc6B) by RT-PCRs using primer pairs F600/lucR1 and F600/LucR2) and Western blots of protein extracts from cells treated with *SNCA* siRNA or valproic acid. RT-PCR generated products (Fig. 3c, lane 2) that were not seen from untransfected cells (Fig. 3c, lane 1). The α-synuclein antibody SYN1 detected a

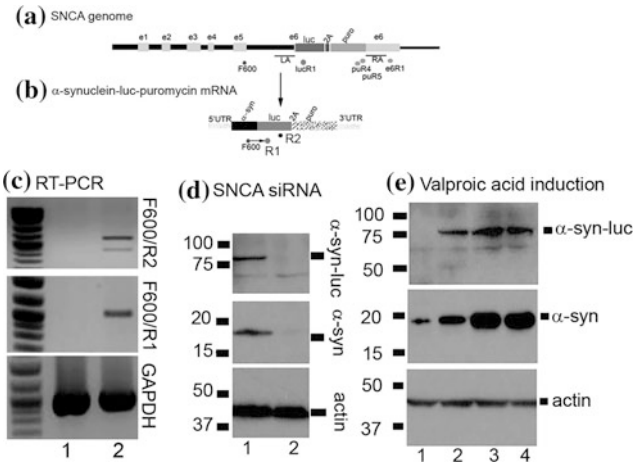

Fig. 3 RT-PCR, Western blot of *SNCA* siRNA treatment LUC6B, and valproic acid induction of α-synuclein expression. **a** Diagram of the modified genome consisting of 6 exons with marker gene (GFP or luciferase) and puromycin resistant gene insert. **b** α-synclein-luciferase (GFP)-2A-puromycin resistant matured mRNA. **c** RT-PCR of untransfected (lane 1) and Luc6B cells (lane 2), **d** control (lane 1) *SNCA* siRNA (lane 2) suppression, and **e** valproic acid induction of α-synuclein expression in Luc6B cells. Lane 1, untransfected cells; lane 2, Luc6B, 0 mM VPA; lane 3, Luc6B, 5 mM; lane 4, Luc6B, 10 mM. Figure was derived from Dansithong et al. 2015 [16]

77 kDA band of the α-synuclein-luciferase (α-syn-luc) fusion protein in cells transfected with a control siRNA (Fig. 3d, lane 1, top) and a18 kDA endogenous α-synuclein. Both the α-synuclein-luc fusion protein and the wild type α-synuclein were not detected when Luc6B cells was transfected with an *SNCA* siRNA (Fig. 3, lane 3).

Valproic acid (VPA) is a histone deacetylase (HDAC) inhibitor that increases the level of histone deacetylation, and hence increases gene expression [17]. VPA has been found to elevate the levels of α-synuclein in SH-SY5Y [18]. Treatment of Luc6B cells with VPA resulted in a dosage dependent increase of both the wild type and modified α-synuclein levels (Fig. 3, lanes 3–4). Detailed methodologies, results, and discussion were described in Dansithong et al. 2015 [16].

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