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J Alzheimers Dis. 2018 ; 66(2): 453–460. doi:10.3233/JAD-180610.**An Inducible Alpha-Synuclein Expressing Neuronal Cell Line Model for Parkinson's Disease¹****Velmarini Vasquez^{a,b,c,e}, Joy Mitra^{a,e}, George Perry^d, K.S. Rao^{b,*}, and Muralidhar L. Hegde^{a,e,f,*}**^aDepartment of Radiation Oncology, Houston Methodist Research Institute, Houston, TX, USA^bCentre for Neuroscience, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología, City of Knowledge, Republic of Panama^cDepartment of Biotechnology, Acharya Nagarjuna University, Guntur, India^dCollege of Sciences, The University of Texas at San Antonio, San Antonio, TX, USA^eHouston Methodist Neurological Institute, Institute of Academic Medicine, Houston Methodist Hospital, Houston, TX, USA^fWeill Cornell Medical College of Cornell University, New York, NY, USA**Abstract**

Altered expression of α -synuclein is linked to Parkinson's disease (PD). A major challenge to explore how the increased α -synuclein affect neurotoxicity is the lack of a suitable human neuronal cell model that mimics this scenario. Its expression in neural precursors affects their differentiation process, in addition to the neuronal adaptability and variability in maintaining a constant level of expression across passages. Here, we describe an SH-SY5Y line harboring Tet-ON *SNCA* cDNA cassette that allows for induction of controlled α -synuclein expression after neuronal differentiation, which can be an important tool for PD research.

Keywords

α -synuclein; dopaminergic neurons; inducible α -synuclein expressing neuronal model for PD; Parkinson's disease

INTRODUCTION

Parkinson's disease (PD), the most common neurodegenerative disorder affecting movement, is characterized by the aggregation/misfolding of α -synuclein in dopaminergic

¹The pCW-FLAG-(inducible)- α -Syn plasmid is being made available in Addgene and the pCW-iFLAG- α -Syn SH-SY5Y cell line is available upon direct request to MLH.

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SUPPLEMENTARY MATERIAL

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neurons. α -synuclein fibrillary inclusions in Lewy bodies and Lewy neurites are the hallmark feature of PD and other synucleinopathies (reviewed in [1, 2]). In familial PD, in addition to various *SNCA* gene mutations [3–5], increased α -synuclein protein levels due to duplication or triplication of the *SNCA* gene [6, 7] has been directly linked to disease progression and severity. Subsequent studies in rodent models that overexpress wildtype or mutant α -synuclein provide strong *in vivo* evidence linking α -synuclein overexpression and PD-like phenotype as well as typical α -synuclein pathology [8, 9]. Similarly, intracerebral administration of preformed α -synuclein fibrils in rodents induces PD-like pathology and movement defects [10–13]. While these cumulative *in vivo* evidence directly links induction of α -synuclein expression to PD pathology, reliable modeling of this linkage in cultured human mature neurons has been challenging.

The majority of *in situ* studies utilize transient or constitutive (stable) overexpression of α -synuclein in neuroblastoma cells followed by their differentiation into dopamine neurons, and recent studies have highlighted the impact of overexpressed α -synuclein on neuronal differentiation [14–17]. In addition, controlling the level of ectopic expression to avoid non-physiologically high levels of expression has been a challenge. Furthermore, induced pluripotent cells (iPSC) derived from autosomal dominant PD patients with *SNCA* triplication (*SNCA*-tri), although showing high α -synuclein levels, their differentiation to neurons is affected by constitutively activated α -synuclein [14, 18]. The study tested prior knockdown of α -synuclein in *SNCA*-tri iPSC cells followed by neuronal differentiation; however, this approach also has limitations of knockdown efficiency, reproducibility as well as timing/controlling α -synuclein expression post differentiation.

Here, we generated and characterized a doxycycline (Dox)-inducible α -synuclein SH-SY5Y cell line, which not only evaded the adverse effects of high α -synuclein levels on differentiation but allowed for controlled, conditional, and reproducible overexpression of α -synuclein before/after neuronal differentiation. We propose that such an inducible neural cell line presents a reliable and more relevant cellular model for PD and other PD-like synucleinopathies, compared to other routinely used cell models.

MATERIALS AND METHODS

Construction of inducible mammalian FLAG- α -Syn expression vector

An inducible mammalian FLAG- α -Syn expression vector was generated by cloning blunt-ended full-length α -Syn amplified from pcDNA-WT- α Syn plasmid using Deep Vent DNA polymerase (M0258, NEB) into a pCW-Cas9 vector (gift from Eric Lander and David Sabatini, Addgene plasmid 50661) [19] and digested with restriction enzymes NheI (R0131, NEB) and BamHI (R0136, NEB) followed by treatment with DNA polymerase I, large (Klenow; M0210, NEB) to generate blunt DNA ends. The primers used to amplify C-terminal FLAG-tagged α -Syn were as follows: forward primer, 5'-ATG GAT GTA TTC ATG AAA GGA CT-3'; reverse primer, 5'-CAC TGT CGA CTT ACT TAT CGT CAT CGT CTT TGT AAT CGG CTT CAG GTT CGT AGT CTT GAT ACC-3'.

Culture and neuronal differentiation of the SH-SY5Y line

The human neuroblastoma SH-SY5Y line was routinely maintained in regular Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Corning). Cells were cultured in Neurobasal medium (Gibco) supplemented with B-27, 1X Glutamax (Gibco), and 1% penicillin/streptomycin (Corning) containing 10 μ M retinoic acid (RA) for seven days to induce neuronal differentiation.

Generation of stably transfected SH-SY5Y line with inducible α -synuclein vector and induction of α -synuclein expression

Transfection of SH-SY5Y cells with pCW-FLAG-(inducible)- α -Syn plasmids was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Dox-inducible α -Syn-expressing cells were selected against the antibiotic puromycin (InvivoGen) with a dose of 10 μ g/mL. Dox (3 μ g/mL for 0, 24, 48, or 72 h)-inducible (i) FLAG- α -Syn levels were optimized for 3–7-fold overexpression.

Cell viability assay

SHSY-5Y cells (4.0×10^6 cells/well) were plated in 96- well plates, and either treated with Dox at different time points or differentiated with RA for seven days followed by Dox treatment. Cell viability was evaluated at specific time points using the CCK-8 kit (CCK- 8; CK04–500, Dojindo Laboratories; Kumamoto, Japan) according to the manufacturer's instructions.

Immunofluorescence microscopy

Cells grown in an 8-well chamber slide were fixed with 4% PFA, permeabilized with 0.2% Triton 100X in 1X PBS and blocked with 5% BSA-PBS-T (1 \times PBS with 0.1% Tween-20), before incubation with primary and secondary antibodies. Oligomer A11 polyclonal antibody (Thermo Scientific, Cat# AHB0052), and α -synuclein antibody LB 509 (Santa Cruz, Cat#sc-58480), ChAT antibody (Cat# AB144P, Millipore) and MAP2 antibody (Cat# GTX11267, Gene Tex) were used. After incubation with Alexa Fluor 488 (green) - and 558 (red)-conjugated secondary antibodies, coverslips were mounted in DAPI (Sigma). Images were taken using an Olympus BX61-Regular Upright BF & Fluorescent/Reflect Microscope using a 60 \times objective. Images were analyzed with Image-J ROI Manager using the free hand selection tool. The corrected total cell fluorescence (CTCF) formula was used to obtain the final fluorescence values: CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

Real time-PCR analysis for mRNA quantitation

Total RNA was isolated using RNeasy Mini Kit (Qiagen #74104). Two microgram of RNA were used for cDNA synthesis in a 20 μ L reaction using SuperScript III reverse transcriptase kit (Invitrogen, #18080–044). α -synuclein expression in the samples was analyzed by SYBR GREEN-based Real Time PCR (7000 Real-Time PCR System; Applied Biosystems) using SYBR Premix Ex Taq (TaKaRa) and primers for targeted mRNA HaSynTfw 5'-AGG GTGTTC TCT ATG TAG G-3' and HaSynTrv 5'-ACT GTC TTC TGG GCT ACT GC-3'

[20]. HPRT expression (internal control) primer sequences used were as follows: forward primer, HPRT-F 5'-TGA CCTTGA TTT ATT TTG CAT ACC-3' reverse primer, HPRT-R 5'-CGA GCA AGA CGT TCA GTC CT-3'. Data were presented as fold change mRNA expression with respect to the reference samples set at 1 based on 2^{-CT} method.

Immunoblotting of cell extracts

Preparation of whole cell extracts and protein quantitation was performed as previously described [21]. Following electrophoresis separation in a 4–16% gradient gel, proteins were transferred onto a PVDF membrane fixed with 0.4% paraformaldehyde (PFA) in 1X PBS for 30 min at room temperature (RT), and blocked with 5% skim milk in 1X TBS-T. The membranes were incubated with primary antibody diluted in 1% skim milk in TBS-T for 1 h at RT or overnight at 4°C, followed by washing in $1 \times$ TBS-T and then incubation with secondary antibody for 1 h at RT and development with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher).

Statistical analysis

Graphpad prism 6 software was used for data analysis. Comparisons of groups were generated with two-way ANOVA followed by Sidak's or Tukey's multiple comparison test to compare selected pair of means. *p*-values are indicated in the associated figure legends.

RESULTS AND DISCUSSION

Transient or constitutive expression of α -synuclein in cultured neural precursor cells and differentiated neurons have been modeled previously to study the biological implications of a high level of α -synuclein expression in neurons. These studies utilized conventional approaches to transfect α -synuclein expression vectors into neural precursor cells or differentiated neurons, using conventional transfection approaches. However, there are inherent limitations and challenges to this approach. First, expression of α -synuclein in neural precursor cells prior to differentiation significantly affects differentiation efficiency [14]. Moreover, such constitutive expression leads to strong cellular adaptability and a progressive decrease in sensitivity to PD-linked etiological factors, contrary to the progressive increase in neuronal dysfunction that is observed in PD. As shown in Supplementary Figure 1A–C, a constitutively overexpressing SH-SY5Y line failed to maintain similar levels of α -synuclein across passages and over time showed loss of overexpression. This was likely due to the mortality of cell population with higher levels of α -synuclein and survival/propagation of cells with moderate/reduced α -synuclein expression. Critically, cellular adaptability to a persistently increased amount of α -synuclein maybe a contributing factor to the loss of toxic effects. Likewise, transfection of differentiated neurons using viral vectors or electroporation is limited by variable efficiency as well as cell toxicity and thus its routine utilization is a challenge [22].

To overcome these limitations, we generated an inducible cell line by stably transfecting SH-SY5Y cells with an α -synuclein cDNA cassette containing a CMV promoter under the control of Tet-on response element (Fig. 1A and Supplementary Figure 1D). The vector also possesses a distal Tet-repressor sequence, which prevented any leaky expression of α -

synuclein in uninduced cells. The stable clone selection using puromycin ensured survival of cells harboring the ectopic α -synuclein cassette. Treatment of pCW-iFLAG- α -Syn SH-SY5Y cells with 3 μ g/mL Dox induced an ~3, 7, and 6-fold increase in FLAG α -synuclein at 24, 48, and 72 h, post induction, respectively (Fig. 1B). We then differentiated the pCW-iFLAG- α -Syn SH-SY5Y cells using retinoic acid [23, 24] for 7 days before inducing with Dox, which again yielded a similar expression pattern (Supplementary Figure 1E). This is consistent with α -synuclein mRNA levels at the same time points (Supplementary Figure 1F). The presence of a Tetrepressor element likely ensures a tight and highly reproducible pattern of protein induction. Notably, comparison of α -synuclein mRNA levels upon Dox-induction, before and after neuronal differentiation revealed that the increase in mRNA levels correlated with α -synuclein protein levels in differentiated neurons, but not in undifferentiated cells. This suggests a cross-talk between α -synuclein levels and neuronal differentiation that coincides with the critical role of α -synuclein in neurons.

Formation of α -synuclein aggregates and inclusion bodies is a key feature of α -synuclein overexpression-mediated neurotoxicity [25, 26]. We observed a progressive increase in α -synuclein oligomers after α -synuclein induction as analyzed by microscopy using an oligomer-specific antibody A11 (Fig. 1C, D). Cell viability was moderately higher in differentiated cells compared to undifferentiated cells overexpressing α -synuclein (Supplementary Figure 1G), possibly due to the replication stress activated upon accumulation of DNA strand breaks induced by the presence of α -synuclein in the chromatin [21].

We next compared the differentiation efficiency before and after α -synuclein induction. Immunofluorescence microscopy of cells for markers of differentiated neurons microtubule-associated protein 2 (MAP2) and choline acetyltransferase (ChAT), clearly showed that α -synuclein induction significantly affected neuronal differentiation (Fig. 2A, B). α -synuclein induction after differentiation did not affect the MAP2 and ChAT levels. Moreover, higher α -synuclein levels during differentiation led to poor neuronal morphology and shorter neurites.

Together, these data underscore the importance of avoiding α -synuclein overexpression during neuronal differentiation. Efficient physiologically relevant differentiation can be achieved with an inducible α -synuclein expressing neural cell line as we show here. The pros and cons of an inducible α -synuclein cell line versus a constitutive and transient expression models is tabulated in Table 1. Our inducible α -synuclein cell line thus provides an ideal system for obtaining mechanistic insights of α -synuclein-mediated neuronal degeneration in PD pathogenesis.

CONCLUSIONS

Modeling of α -synuclein pathology in cellular and animal models that closely represent key aspects of human PD has been a fundamental challenge in understanding the molecular pathways involved in α -synuclein induced neurotoxicity in PD and in exploring ways to ameliorate these pathways. The early promise of PD patient-derived iPSC cells with (*SNCA*-tri), although, consistently shows higher α -synuclein levels and many aspects of

neurotoxicity, the constitutively increased α -synuclein limits its faithful neuronal differentiation [14]. The limitations of existing models in presenting key features of human synucleinopathies together with limited tight-reproducibility of the underlying pathology poses a significant challenge for translating the *in-cellulo* findings to *in vivo* models and human PD. From this perspective, the Dox-inducible pCW-iFLAG- α -Syn SH-SY5Y line reported here, allows ectopic expression of α -synuclein in a controlled and reproducible fashion. An important advantage of this stable cell line is its post-differentiation induction of expression, thereby preventing any non-physiological protein expressions during the differentiation process. Although, a neuroblastoma line was reported here as a proof-of-concept to demonstrate the advantages of an inducible α -synuclein expression system, a similar approach can be utilized to generate iPSC or neural progenitor cell line, which can be differentiated to various neuronal types before α -synuclein induction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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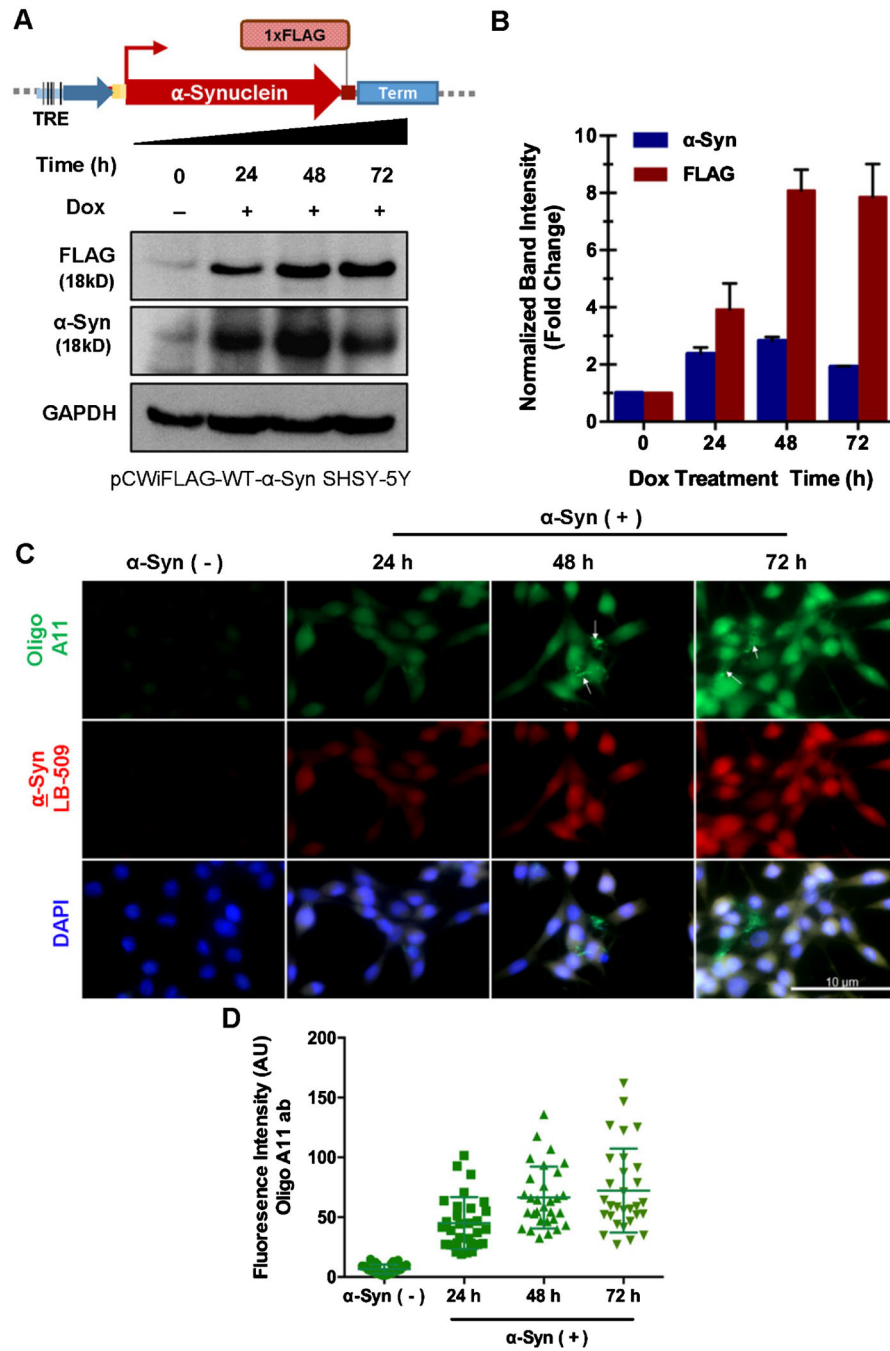


Fig. 1. Generation and characterization of inducible α -synuclein (α -Syn) expressing neuronal line. A) Schematic representation of doxycycline-inducible pCW-iFLAG- α -Syn vector used to generate SHSY-5Y stable cell line and immunoblot showing time-dependent induction of FLAG α -synuclein. B) Densitometry analysis of FLAG and endogenous α -synuclein immunocontent normalized to GAPDH. C) Immunofluorescence of oligomer conformations upon α -synuclein expression. Anti-oligomer A11 antibody recognizes all types of

oligomers, but not monomers and fibrils in the case of α -synuclein. D) Oligo A11 antibody fluorescence intensity per cells. Measurement from 30 cells from three different fields.

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Table 1

Pros and Cons of inducible α -synuclein expression over transiently or constitutive α -synuclein expressing lines

Inducible	Constitutive	Transient
Not limited by transfection efficiency	Not limited by transfection efficiency	Limited by transfection efficiency
Controlled overexpression that is unaffected across cell passages	Controlled overexpression for a limited number of passages due to mortality of overexpressing cells	Short time overexpression, whose efficiency/consistency varies from experiment to experiment
Allows for the comparison of α -Syn effect in the same cell lineage (i.e., treated vs. untreated with Dox)	A cell line with no α -Syn overexpression is needed as a control	Allows for the comparison of α -Syn effect in the same cell lineage (i.e., transfected with α -Syn vector vs. transfected with empty vector).
α -Syn overexpression can be induced after differentiation to avoid α -Syn effect on differentiation	Differentiation is affected by the presence of α -Syn	Viral transductions or electroporation are required for overexpressing α -Syn, which are limited by transfection efficiency and toxicity

Green shades indicate pros and cells in orange shade indicate cons.