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14. ABSTRACT
Background: iPS reprogramming to model 'disease-in-a-dish has become an attractive approach to study disease mechanisms. However, a frank pathophysiological phenotype in iPSC-derived PD neurons remains to be shown.
Objective/Hypothesis: The objective of this proposal is to accelerate the aging process of iPS-derived dopaminergic neurons with the goal of reproducing a Parkinson's disease (PD) specific phenotype in vitro.
Specific Aim 1.
To generate vector constructs and to establish Dox-inducible iPSC lines that express the Hutchinson-Gilford progeria (HGPS) gene (mutant lamin A with an in-frame loss of 150 nucleotides) and differentiate them into dopaminergic neurons.
Deliverable of this aim is the introduction of mutant lamin A in iPSC lines and optimization of inducible expression of mutant lamin A in iPSCs and during the differentiation into dopaminergic neurons.
Specific Aim 2.
To test whether iPSC-derived dopaminergic neurons transduced with mutant lamin A show changes in age-regulated genes at the mRNA and protein levels at different time-points during differentiation, thus exhibiting an accelerated aging.
Deliverable of this aim is the assessment of specific age-related aging pattern of gene and protein expression in induced mutant lamin A modified iPSC-derived neurons.
Specific Aim 3.
To test whether induced mutant lamin A cell lines differentiated into dopaminergic neurons exhibit hallmark pathology of PD, such as protein aggregation of alpha-synuclein, posttranslational modification, and signs of mitochondrial pathology.
Deliverable of this aim is the assessment of the pathological PD-related phenotype in induced mutant lamin A modified iPSC-derived neurons.
Study Design: This is an in vitro study of patient-specific iPSC-derived dopaminergic neurons in which truncated lamin A will be introduced to study cellular phenotypes under the hypothesis that cells under expression of lamin A will age faster than untreated cells.
Relevance: Creation of iPSC lines from patients with PD that develop a disease phenotype would revolutionize research in PD and could produce more predictive disease models to enable the advancement of better candidates into clinical testing. If successful, the impact of this research project on the iPSC field could be enormous. It could remove one of the remaining roadblocks to using iPSC model for PD for the study of disease mechanism and drug development, which could bring us closer to finding the cause and cure for PD.

15. SUBJECT TERMS
Parkinson's disease, induced pluripotent stem cells, cellular model, accelerated aging, lamin A, progerin

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Final Report for grant W81XWH-12-1-0003

Title: "Induced accelerated aging in induced pluripotent stem cell lines from patients with Parkinson's disease", Principal Investigator: Birgitt Schuele, MD

Table of contents:

A. Introduction:

The overall goal of the study was to develop a cell culture system from patient-derived induced pluripotent stem cells that expresses genes that can accelerate the aging process to facilitate modeling of neurodegenerative diseases such as Parkinson's disease. The *objective of this study* was to introduce the truncated lamin A gene, progerin, into patient-derived induced pluripotent stem cells using a doxycycline (Dox)-inducible expression system and differentiate them into dopaminergic neurons with the goal to accelerate the aging process of these cells and to promote a PD-related phenotype thus establishing a 'disease-in-a-dish' model for PD. During our study, a publication from Lorenz Studer showed a very similar approach with lentiviral constructs of truncated lamin A in iPSC-differentiated neurons (Miller et al. 2013). It shows that our hypothesis is correct and that the introduction of mutant forms of lamin A that can cause Hutchinson Gilford Progeria syndrome is accelerating the neurodegenerative phenotype in vitro.

We also improved our differentiation protocol for developing glia cultures, specifically oligodendrocyte cultures. Oligodendrocyte dysfunction has been described as a major factor in the process of initiation and progression of neurodegeneration also for parkinsonian syndromes such as multiple system atrophy. We have been optimizing various culture conditions from different publicly available culture protocols and have now developed a protocol that allows for robust differentiation into oligoprecursor cultures.

B. Body

Progress

1. Development of RetroX Tet-on system for progerin expression in iPS-derived cultures. Overall, we had major challenges to transduce patient-derived induced pluripotent stem cells (iPSC) with the RetroX Tet-on system and to isolate clones that would have both constructs incorporated the activator rtTA and the GFP-Progerin construct. Human iPSCs can be difficult to transduce and obtain high efficiency. Because of these challenges, we decided to change the strategy and transduced induced pluripotent stem cell-derived neural stem cells (NSCs) from different patients with the constructs and to enrich the cell population by Fluorescence-activated cell sorting (FACS) (Appendix 1,

experimental results of different FACS sorting experiments). With large numbers of neural stem cells, we achieved reasonable transduction of neural stem cells, however, the major challenge was that the neural stem spontaneously differentiated after transduction and FACS sorting, which could have been caused by the stress of the cell manipulation or the expression of the lamin A construct itself in these cells. Under physiological conditions, lamin A is not expressed at these early stages of development and the expression of mutant lamin A could have caused the differentiation and subsequent cell death. Therefore the approach by Miller et al. to introduce progerin on neurons might be the better approach to age the cells. We had envisioned generating an inducible system to be able to study the effects of progerin at defined timepoints, but the induced spontaneous differentiation and subsequent cell death after manipulation of the cells prohibited this approach.

2. Differentiation into oligoprecursor cells (OPCs) and oligodendrocytes

As we have developed a robust dopaminergic differentiation protocol in our lab (Mak et al. 2012), we also wanted to derive oligodendrocytes, since oligodendrocytes are also implicated in the development of neurodegeneration. We made progress deriving oligoprecursor cells from neural stem cells and in the process of differentiating them into oligodendrocytes (Appendix 2, A sequential differentiation approach to generate oligodendrocytes from human iPSCs derived neural stem cells). We compared three different differentiation protocols and developed a protocol that can provides a >90% oligoprecursors. We also extensively assessed expression of neuronal and glial markers in these cultures and defined a novel set of markers MOG, MBP, and NKX2.2 that can distinguish between neural stem cells and oligoprecursors whereas other reported marker overlap largely with neural stem cells, e.g. Olig 1/2.

C. Key Research Accomplishments:

- *Milestone 1* (month 1-2): generation of the drug-inducible construct using Clontech Retro-X Tet-On advanced vector **COMPLETED**

- *Milestone 2* (month 2-3): establishing stable iPSC clones and NSC clones with drug-inducible progerin construct **NOT COMPLETED DUE TO TECHNICAL DIFFICULTIES**
- *Milestone 3* (month 4-6): differentiation into DA neurons **COMPLETED,** differentiation into oligodendrocytes, **COMPLETED**
- *Milestones 4* (month 6-12): examination of DA-phenotype, age-related phenotype, and pathological phenotype related to PD **NOT COMPLETED DUE TO TECHNICAL DIFFICULTIES IN MILESTONE 2**

D. Reportable Outcomes:

At this point, no publications or posters have been derived from the grant.

E. Conclusion:

The derivation of induced pluripotent stem cell lines or neural stem cells expressing an inducible progerin gene to accelerate the aging process was in our hands not successful mainly due to the fact the cells did not survive the transduction and selection process and spontaneously differentiated.

We made however, progress towards improving our differentiation methods for oligodendrocytes and identified a new set of markers for oligoprecursor cells.

We had requested a no cost extension of the project to continue on this project, however, but with the challenges at the experimental level for the main goal of the grant that we could not overcome, we will return the remaining funds to the funding agency.

F. References:

Miller, J.D., Ganat, Y.M., Kishinevsky, S., Bowman, R.L., Liu, B., Tu, E.Y., Mandal, P.K., Vera, E., Shim, J.W., Kriks, S., *et al.* (2013). Human iPSC-Based Modeling of Late-Onset Disease via Progerin-Induced Aging. Cell stem cell *13*, 691-705.

Hu BY, Du ZW, Zhang SC: Differentiation of human oligodendrocytes from pluripotent stem cells, Nat Protoc 2009, 4:1614-1622

Sharla M. O. Phipps, Joel B. Berletch, Lucy G. Andrews, and Trygve O. Tollefsbol:

Aging Cell Culture: Methods and Observations, Methods Mol Biol. 2007 ; 371: 9–19

G. Appendices:

Appendix 1: Experimental results of FACS sorting experiments

Appendix 2: A sequential differentiation approach to generate oligodendrocytes from human iPSCs derived neural stem cells

Appendix 1:

Generation of stable hNSC cell lines expressing inducible GFP-Progerin by retroviral transduction

Jing Bian, PhD and Birgitt Schuele, MD

Objective: To derive stable NSC cell lines (Huf5, Huf4 and 1857) expressing doxycycline inducible GFP-Progerin

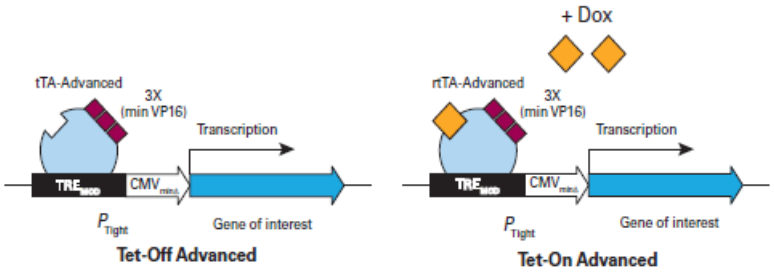


Figure 1. Gene induction in the Tet-Off Advanced and Tet-On Advanced Systems. The Tet-controlled transactivators for these systems (tTA-Advanced and rtTA-Advanced) are fusion proteins derived from a wild-type or mutant Tet repressor (TetR), respectively. Each TetR DNA-binding domain is joined to three minimal transcription activation domains which activate transcription when bound to the tetracycline response element (TRE) in P_{Tight}. For Tet-Off Advanced, removal of doxycycline permits tight binding and induces high-level transcription. In exact contrast, the Tet-On Advanced System is activated by doxycycline.

When we transduced the hNSC cell lines with co-infection of retrovirus pRetro-X-Tet-on and pRetro-X-GFP-Progerin and induced with Dox containing medium, we will observe the green fluorescence expressed in the hNSC cell lines.

Experimental protocol:

Prepare stock solution: Polybrene (Millipore, Cat. No. TR-1003-G) 10 mg/ml, Doxycycline (Clontech, REF 631311)10 mg/ml, Retroviral MOI of 0.5 particle per cell is used. (Retrovirus MOI is $1*10^5$ particles /ul)

1. $2*10^5$ hNSCs are plated in each well of 12 well plate
2. The next day when the cell confluency is 80-90%, we start retroviral infection
3. Chang NSC medium containing 4 ug/ml polybrene in each well of 12 well plate
4. Add 1 ul of each corresponding retrovirus in each well as assigned in Table 1
5. Change fresh NSC medium containing 500 ng/ml Doxycycline after overnight retrovirus incubation
6. After another day of Doxycycline induction, for the cells with co-infection of pRetroX-Tet-on and pRetroX-GFP-Progerin), we bring the plate under the fluorescence microscope to observe green fluorescence. (Figure 2)
7. For single retrovirus transduction, we will continue selection with G418 (for pRetroX-Tet-on) and puromycin (for pRetroX-GFP-progerin).

Table 1. Retroviral transduction in three NSC cell lines (1857, Huf4, and Huf5)

	1857 Coinfection of pRetro-x-GFP-Progerin and pRetro-x-Tet-on	Hu4 Coinfection of pRetro-x-GFP-Progerin and pRetro-x-Tet-on	Huf5 Coinfection of pRetro-x-GFP-Progerin and pRetro-x-Tet-on	
	1857	Huf4	Huf5	

	Infection of pRetro-x-Tet-on	Infection of pRetro-x-Tet-on	Infection of pRetro-x-Tet-on	
	1857 Infection of pRetro-x-GFP-Progerin	Huf4 Infection of pRetro-x-GFP-Progerin	Huf5 Infection of pRetro-x-GFP-Progerin	

2) Second Retrovirus infection

A. Fluorescence image of GFP-Progerin expression in three NSC cell lines

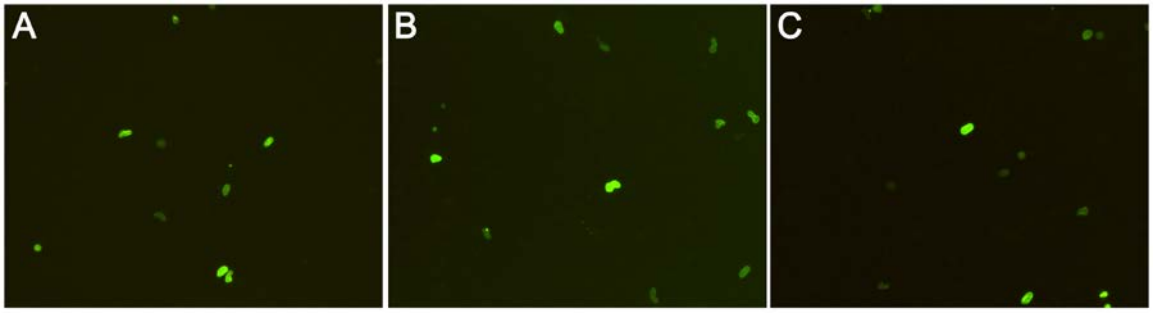
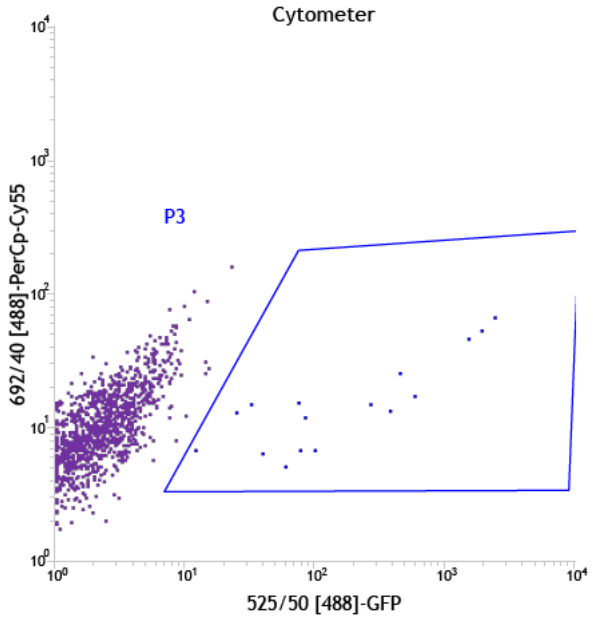


Figure 3. Second time of Retrovirus co-infection of pRetroX-Tet-on and pRetroX-GFP-Progerin into three hNSC cell lines 1857 (A), Huf4 (B) and Huf5 (C) with Dox induction for 24 hours

B. Flow sorting report for GFP positive for each NSC cell line after retrovirus infection with Dox induction is as folllowing.

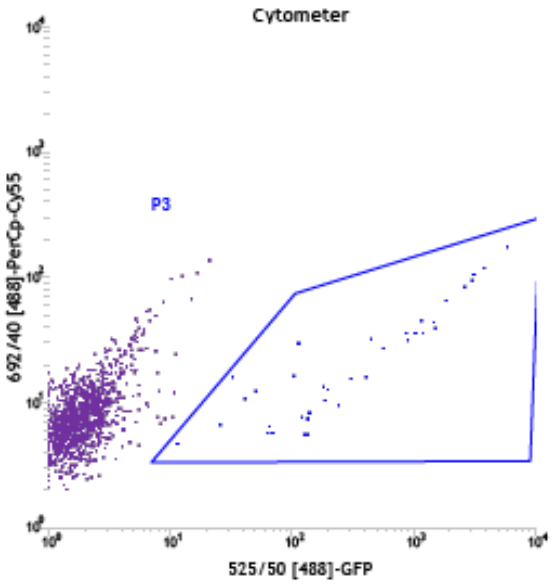
a) Flow sorting of 1857 NSC GFP-progerin positive cells after Retrovirus infection with Dox-induction



Statistics: Cytometer

Populations	Events	% Total	% Parent	525/50... Mean	692/40... Mean
All Events	2,000	100.00%	####	8	12
P1	1,192	59.60%	59.60%	13	14
P2	1,079	53.95%	90.52%	10	13
P3	16	0.80%	1.48%	509	20
NOT(P1)	808	40.40%	40.40%	3	10

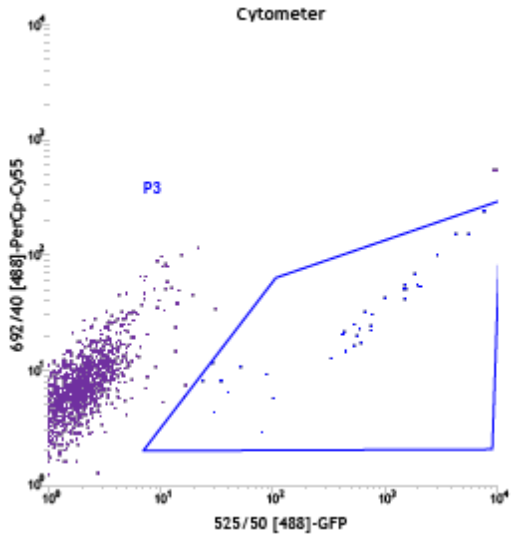
b) Flow sorting of Huf4 NSC GFP-progerin positive cells after Retrovirus infection with Dox-induction



Statistics: Cytometer

Populations	Events	% Total	% Parent	525/50... Mean	692/40... Mean
All Events	2,000	100.00%	####	19	8
P1	1,278	63.90%	63.90%	28	10
P2	1,175	58.75%	91.94%	30	10
P3	36	1.80%	3.06%	912	33
NOT(P1)	722	36.10%	36.10%	2	5

c) Flow sorting of Huf5 NSC GFP-progerin positive cells after Retrovirus infection with Dox-induction



Statistics: Cytometer

Populations	Events	% Total	% Parent	525/50... Mean	692/40... Mean
All Events	2,000	100.00%	####	30	8
P1	1,151	57.55%	57.55%	50	12
P2	1,085	54.25%	94.27%	50	11
P3	34	1.70%	3.13%	1,236	41
NOT(P1)	849	42.45%	42.45%	2	4

3) Third Retrovirus infection

A. Fluorescence image of GFP-Progerin expression in three NSC cell line

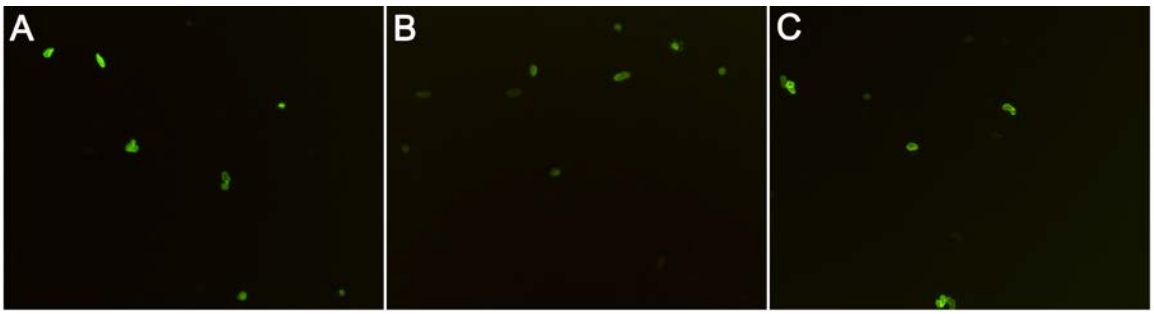
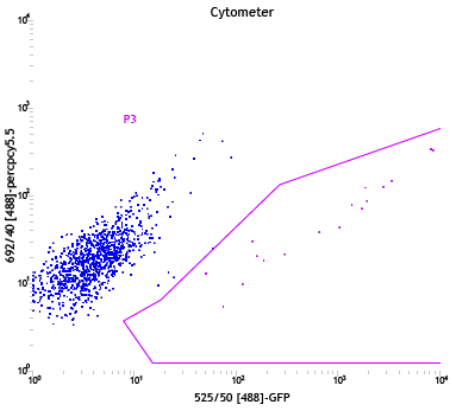


Figure 4. Third time of Retrovirus co-infection of pRetroX-Tet-on and pRetroX-GFP-Progerin into three hNSC cell lines 1857 (A), Huf4 (B) and Huf5 (C) with Dox induction for 24 hours

B. Flow sorting report for GFP positive for each NSC cell line after retrovirus infection with Dox induction is as folllowing.

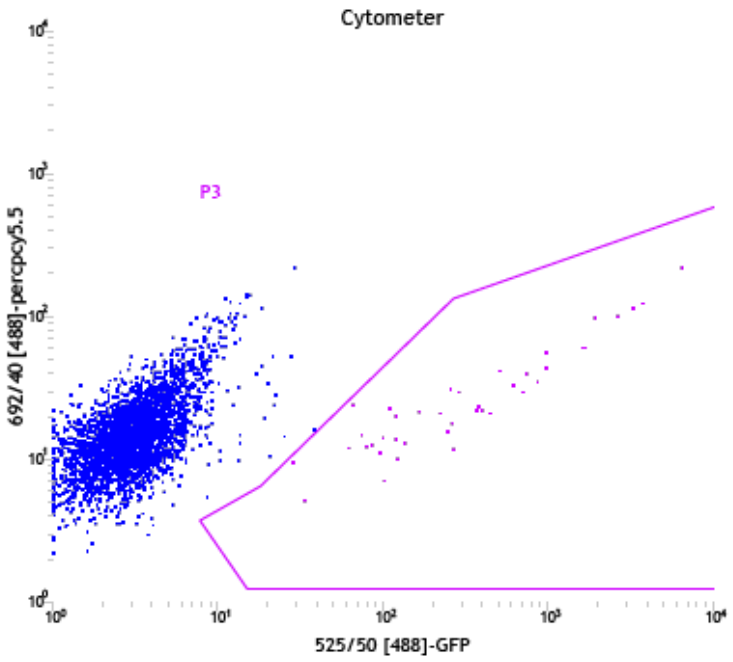
a) Flow sorting of 1857 NSC GFP-progerin positive cells after Retrovirus infection with Dox-induction



Populations: Cytometer

Populations	Events	% Total	% Parent
All Events	2,000	100.00%	####
P1	1,226	61.30%	61.30%
P2	1,099	54.95%	89.64%
P3	17	0.85%	1.55%
NOT(P1)	774	38.70%	38.70%

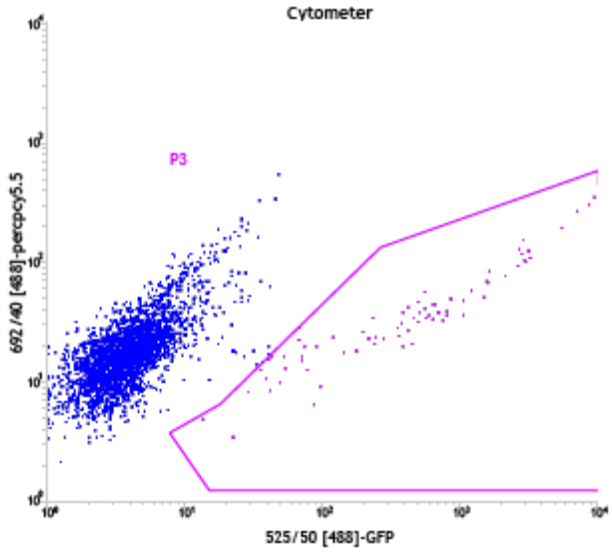
b) Flow sorting of Huf4 NSC GFP-progerin positive cells after Retrovirus infection with Dox-induction

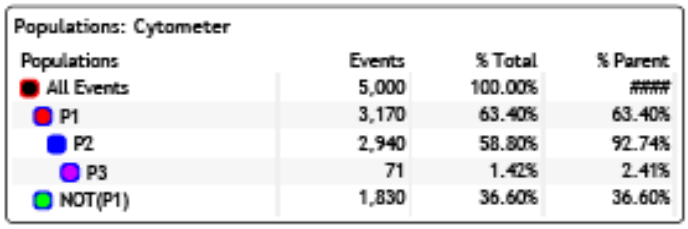


c) Flow sorting of Huf5 NSC GFP-progerin positive cells after Retrovirus infection with Dox-induction

Populations: Cytometer

Populations	Events	% Total	% Parent
All Events	5,000	100.00%	####
P1	3,400	68.00%	68.00%
P2	3,231	64.62%	95.03%
P3	40	0.80%	1.24%
NOT(P1)	1,600	32.00%	32.00%





Populations: Cytometer

Populations	Events	% Total	% Parent
All Events	5,000	100.00%	#####
P1	3,170	63.40%	63.40%
P2	2,940	58.80%	92.74%
P3	71	1.42%	2.41%
NOT(P1)	1,830	36.60%	36.60%

C. Quantification of GFP-progerin positive in all three NSC cell lines after third time retroviral infection

Table 2. Quantification of GFP-progerin positive in 3 NSC cells lines after third retrovirus infection

Name	population	Event Counts	Sort Count
1857	P3	3,495,167	20,488
Huf4	P3	10,121,921	67,506
Huf5	P3	6,626,754	47,018

4/1/2014
Experimental protocol:

Prepare stock solution: Polybrene (Millipore, Cat. No. TR-1003-G) 10 mg/ml, Doxycycline (Clontech, REF 631311)10 mg/ml, Retroviral MOI of 0.2 particle per cell is used. (Retrovirus MOI is $1*10^5$ particles /ul)

$1*10^6$ hNSCs are plated in each well of 6 well plate
The next day when the cell confluency is 80-90%, we start retroviral infection
Chang NSC medium containing 4 ug/ml polybrene in each well of 6 well plate
Add 1 ul of pRetro-Tet-on and 1 ul of pRetro-GFP-Progerin retrovirus in each well as assigned in Table 1
Change fresh NSC medium after 18 hours of retro-virus incubation, we observed 80% cell death after retrovirus infection. (Figure 1)
48 hours after retrovirus infection, change NSC medium containing 500 ng/ml Doxycycline
After incubating NSCs with Doxycycline for 24 hours, fluorescence image will be taken And GFP positive NSC cells will be collected with sorting with filter cap(BD 3265807) and transfer to sorting tube (BD 3265807).

Table 1. Retroviral transduction in three NSC cell lines (1857, Huf4, and Huf5)

1857 Coinfection of pRetro-x-GFP-Progerin and pRetro-x-Tet-on	Hu4 Coinfection of pRetro-x-GFP-Progerin and pRetro-x-Tet-on	Huf5 Coinfection of pRetro-x-GFP-Progerin and pRetro-x-Tet-on
duplicate	duplicate	duplicate

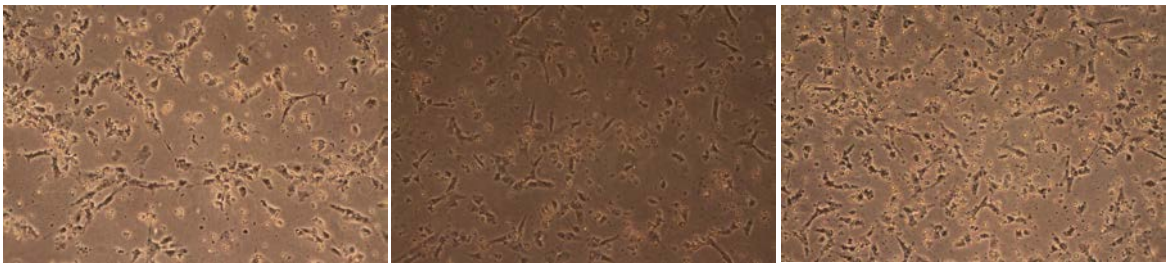
Experiment Result:

2. NSC cell lines progerin-GFP expression after infection of pRetroX-Tet-on and pRetroX-GFP-progerin with Dox induction

24 hours after retrovirus infection of pRetroX-Tet-on and pRetroX-GFP-Progerin into three hNSC cell lines 1857 , Huf4 and Huf5, and only Huf5 was observed GFP positive

Experiment modification

1. Change retrovirus dosage, 1.5 ul of Retro-Tet-on + 1.5 ul of Retro-Progerin-GFP or 2 ul of Retro-Tet-on + 2 ul of Retro-Progerin-GFP in each well of 6-well plate
2. Due to the frequent thawing and freezing, the tranduction efficiency of each vial of retrovirus was reduced, so we did first day and second day retrovirus infection.



1857 Huf4 Huf5

After retrovirus infection, 60% of all three NSC cells died and the remaining NSCs differentiated into neuronal-like cells.

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The Parkinson's Institute and Clinical Center

Appendix 2

A sequential differentiation approach to generate oligodendrocytes from human iPSCs derived neural stem cells

Jing Bian, PhD and Birgitt Schuele, MD

Development of expandable OPCs from human iPSC derived NSCs

We developed an efficient differentiation protocol to differentiate hiPSC derived NSCs into oligodendrocytes and compared three different protocols for oligodendrocyte differentiation. The schematic (Figure 1) below shows the timeline with the different supplements and recombinant proteins of our optimized protocol.

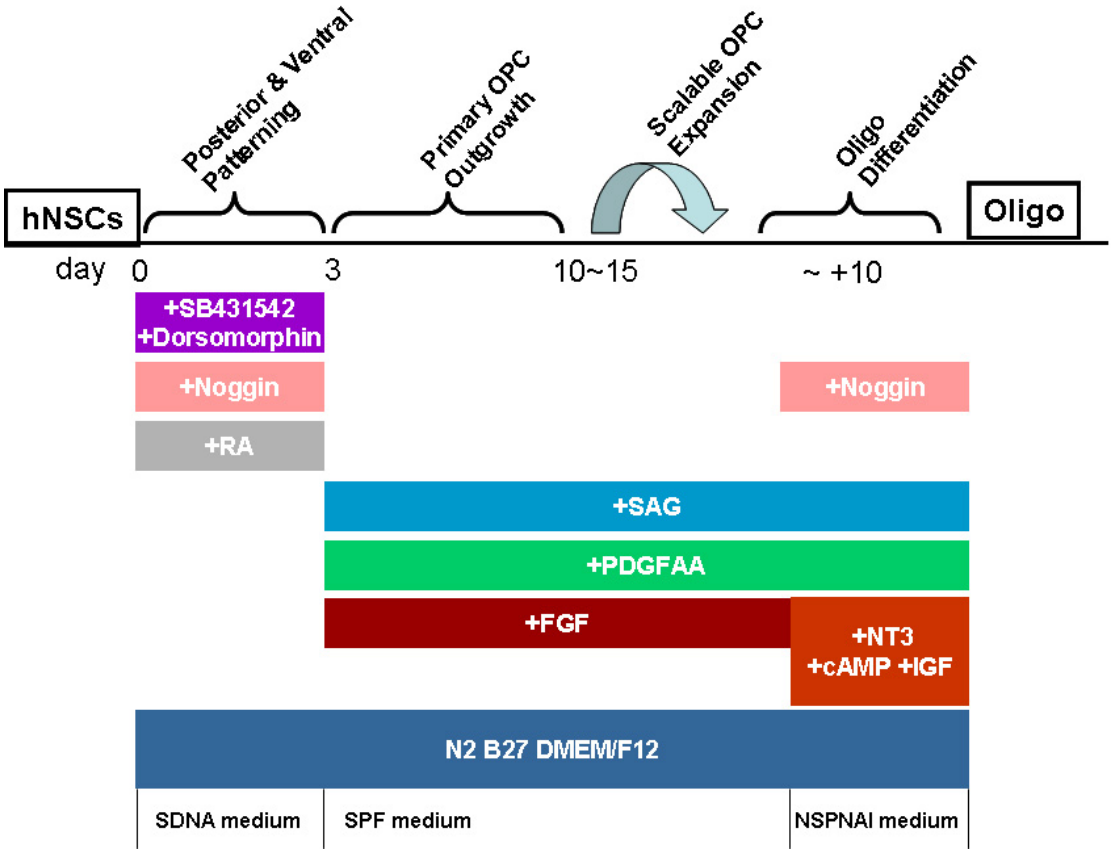

Figure 1. Defined developmental cues induce the rapid differentiation of hiPS derived NSCs into oligodendrocytes. Schematic time course showing the factors used to differentiate hiPS derived NSCs through a series of developmental transitions into functional oligodendrocytes.

We were able to derive oligoprecursors from three different human iPSCs with similar efficiencies as shown in Figure 2.

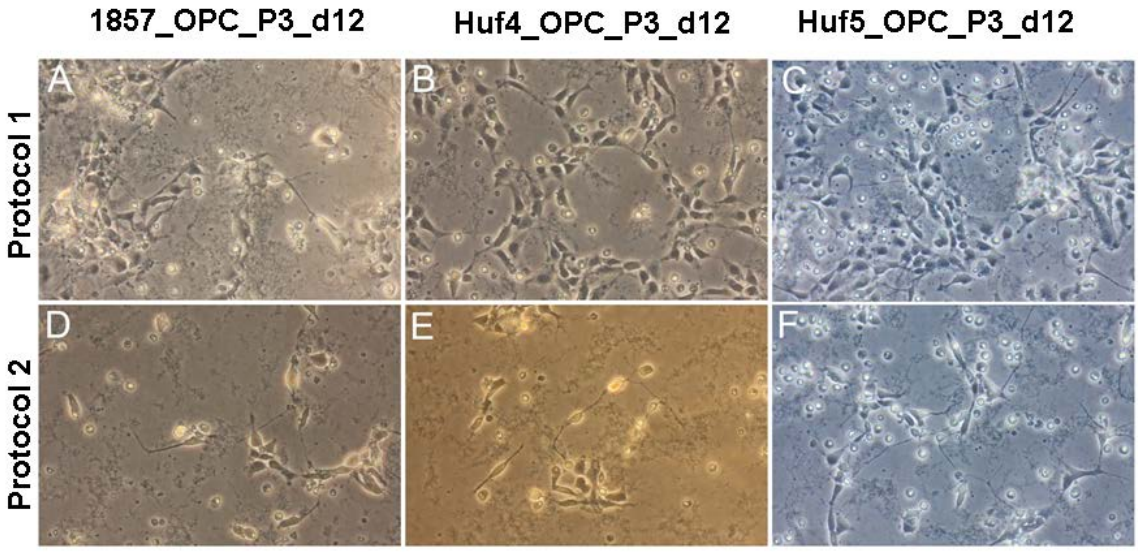

Figure 2. Oligoprecursors from three different hiPSCs. Comparison of two published protocols.

Next, we assessed expression at different stages during oligodifferentiation on a 47 Taqman gene expression array and compared expression to undifferentiated neural stem cells. We detected three markers to be highly expression in these cultures, such as MBP, MOG, and NKx2.2. These markers have not been described before as specific to oligoprecursors as a signature. We are currently repeating these experiments.

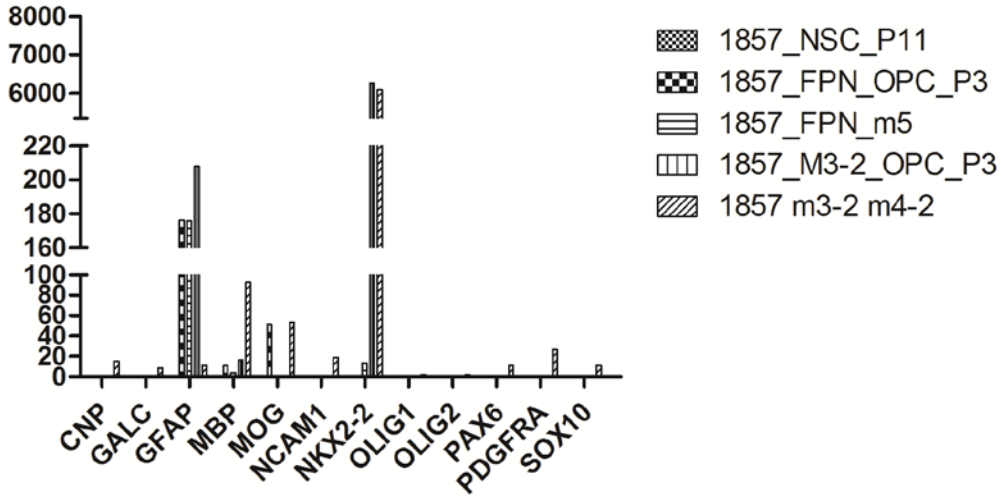

Figure 3. Gene expression graph: RQ plot, Sample set 2 Log fold expression change relative to Huf5 NSC p25 Control 200K

	GFAP	A2B5	NG2	PDGFR-α	MOG	MAG	MBP	OLIG-1	OLIG-2	NKX2.2	SOX10
Derived from adult NSC (Neri et al., 2010)	+				+	+	+				
Human iPSC derived OPCs and Oligos (Wang et al., 2013)				+					+	+	+
mEpsc derived OPCs and Oligos (Najm et al., 2011)		+	+	+				+	+	+	+
OPC markers in human brain (Chang et al., 2000; Windrem et al., 2004)		+	+				+				
OPCs from hiPSC derived NSCs from our lab	+				+		+			+	

Table 1. Summary of OPC markers we detected as a signature specific to distinguish between NSCs and OPCs based on gene expression.