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Generating Neural Stem Cells from iPSCs with Dopaminergic Neurons Reporter Gene

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Abstract

Genetic reporters offer attractive approaches to investigate gene expression, gene function, and spatiotemporal assessment in vitro and in vivo. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the biosynthesis of the dopamine neurotransmitter, and thus it has been used as a reliable marker for dopaminergic neurons in vitro and in vivo. Herein we describe a method for making iPSC lines with TH-green fluorescent protein reporter gene using CRISPR/Cas9 technique.

Keywords

Tyrosine hydroxylase; Reporter; CRISPR/Cas9; Human pluripotent stem cells; Neural stem cell; Parkinson's disease

1 Introduction

Dopaminergic (DA) neurons are involved in many critical functions within the central nervous system (CNS), and dopamine neuro-transmission impairment underlies a wide range of disorders from motor control deficiencies, such as Parkinson's disease (PD) [1], to psychiatric disorders, such as alcoholism, drug addictions, bipolar disorders, and depression. Neural stem cell-based technology has potential to play an important role in developing efficacious biological and small molecule therapeutic products for dopamine dysregulation disorders. Tracking DA neurons with a reporter gene in real-time live cell culture system or brain tissue is an attractive and sound approach to study their development, physiology, and pathophysiology. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the biosynthesis of dopamine. Thus, it has been used as a reliable marker for DA neurons [2].

Induced pluripotent stem cells (iPSCs) are generated from adult somatic cells through the introduction of several transcription factors [3, 4]. iPSCs may be used to derive patient's own cells and model diseases, such as Parkinson's disease in vitro [5, 6]. The combination of iPSC-based in vitro models and the TH gene reporter approach will enable reliable systems for investigating dopaminergic neurons derived from healthy controls or patients with disease.

Herein, we describe a method for establishing TH-derived green fluorescent protein-expressing reporter in human iPSCs using CRISPR/Cas9 technique [7–11]

2 Materials

2.1 Construction of gRNA Expressing Vector and Donor Vector

1. pSpCas9(BB)-2A-GFP (pX458) (Addgene).
2. AAVS1 hPGK-PuroR-pA donor (Addgene) [8].
3. 5 M NaCl.
4. Polymerase chain reaction (PCR) machine.
5. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.0 BbsI (New England BioLabs).
6. QIAquick Gel Extraction Kit (QIAGEN).
7. T4 ligase and buffer (New England BioLabs).
8. Gibson Assembly Master Mix (New England BioLabs).
9. One Shot Stbl3 Chemically Competent *E. coli* (Invitrogen).

2.2 Transfection of Plasmid into HEK293FT Cells and Verification of gRNA Using T7 Endonuclease 1

PureLink Quick Plasmid Miniprep Kit (Invitrogen).

HEK 293FT cells (Life Technologies).

DPBS, no calcium, no magnesium (Gibco).

0.05% Trypsin-EDTA (Gibco).

10% FBS media: Prepare DMEM, high glucose (Gibco) by supplementing 10% HyClone FetalClone II Serum (GE Healthcare Life Sciences). Store at – 4 °C.

Corning 60 mm TC-Treated Culture Dish (Corning).

Opti-MEM, Reduced Serum Medium (Gibco).

Lipofectamine 2000 transfection reagent (Life Technologies).

T7 Endonuclease 1 (T7E1, New England BioLabs).

Lysis buffer for genomic DNA isolation: 1 M Tris–Cl, 0.5 M EDTA, 5 M NaCl, 10% SDS.

2.3 Nucleofection of Vectors into hiPSCs in a Feeder-Free Condition

Human recombinant Laminin-521 (BioLamina).

DPBS, calcium, magnesium (Gibco).

StemFlex (Gibco): Mix StemFlex Basal Medium with StemFlex supplement, and divide into aliquots. Store at -20°C .

Corning 100 mm TC-Treated Culture Dishes (Corning).

Puromycin-Resistant Mouse Embryonic Fibroblasts, Day E13.5 (STEMCELL Technologies).

DPBS, no calcium, no magnesium (Gibco).

0.4 mg/mL Mitomycin C (ACROS ORGANICS): Dissolve 2 mg in 5 mL of prewarmed DPBS, no calcium, no magnesium.

0.05% Trypsin-EDTA (Gibco).

10% FBS media.

Accutase cell detachment solution (STEMCELL Technologies).

Nucleofector 2b (Lonza).

Human Stem Cell Nucleofector Kit 1 (Lonza).

Rho-associated protein kinase (ROCK) inhibitor (Y-27632, Millipore).

NHEJ inhibitor-SCR7 (Xcess Biosciences Inc.) [9].

Puromycin dihydrochloride (Life Technologies).

2.4 Neural Stem Cell Differentiation

Disposable Cell Lifter (Fisher Scientific).

20 $\mu\text{g/mL}$ human recombinant basic fibroblast growth factor (bFGF): Dissolve 50 μg in 2.5 mL of 10 mM Tris base buffer, pH 7.6, and divide into aliquots. Store at -20°C .

100 $\mu\text{g/mL}$ human recombinant epidermal growth factor (EGF): Dissolve 500 μg in 5 mL of 10 mM Tris base buffer, pH 7.6, and divide into aliquots. Store at -20°C .

NSC media: Prepare 50 mL of NN1 media (NeoNeuron), and add 50 μL of 20 $\mu\text{g/mL}$ bFGF (final 20 ng/mL) and 10 μL of 100 $\mu\text{g/mL}$ EGF (final 20 ng/mL). Store at -4°C .

Corning 60 mm TC-Treated Culture Dish (Corning).

0.05% Trypsin-EDTA (Gibco).

Trypsin neutralizer solution (Gibco).

Corning 24 well TC-Treated Multiple Well Plates (Corning).

10 $\times$ poly-L-ornithine: Prepare 0.16 mg/mL of poly-L-ornithine as 10 times concentrated, and divide into aliquots. Store at -20°C .

Differentiation of dopaminergic neurons: Prepare 25% of NN1 media (NeoNeuron) and 75% of DIF1 (NeoNeuron), 10 ng/mL of bFGF, 200 mM ascorbic acid, and 1% of FBS. Store at 0° C (*see* Note 1).

3 Methods

3.1 Construction of gRNA Expressing Vector and Donor Vector

1. Construction of gRNA Expressing Vector.
 - a. Search the candidate gRNAs using online gRNA searching website, such as CRISPRdirect [10] or refer to published paper (*see* Note 2).
 - b. Order gRNA oligonucleotides with additional sequences for the connection with BbsI cleaved ends of backbone plasmid.
Forward: CACC (20 bp gRNA sequences) CT.
Reverse: AAACAG (20 bp reversed gRNA sequences)
 - c. Annealing the gRNA Oligonucleotides Using PCR.
Mix 24.5 µL of each oligonucleotide (100 µM) with 1 µL of 5 M NaCl, and total volume is 50 µL. Set the mixture on the PCR machine and annealing by operation as 99° C 5 min, 95° C 5 min and –5° C every 5 min to 20° C, and hold at 4° C. Dilute the annealed oligonucleotides 1000 times with TE buffer.
 - d. Ligation to pX458 Plasmid.
Linearize pX458 by BbsI and purify by electrophoresis and gel extraction. Mix 2 µL of the annealed oligonucleotides, appropriate amount of linearized pX458, 10XT4 ligase buffer, and T4 DNA ligase, and react on an ambient temperature for 1 h. Apply into a competent cell for the transfection, and confirm the insertion of gRNA sequences by sequencing.
2. Construction of Donor Vector for Homologous Recombination.
 - a. Linearize AAVS1-hPGK-PuroR-pA donor plasmid with Pme I and EcoR I, and extract backbone plasmid by electrophoresis-gel extraction.
 - b. Prepare wild-type genomic DNA (*see* Note 3) and pX458 plasmid as PCR template to clone homologous arms and T2A-GFP-pA. Set the PCR primers having additional 16 bp homologous sequences with the linearized AAVS1 hPGK-PuroR-pA donor plasmid at the 5' end (Fig. 1, dotted × line above a forward and c reverse primer).

¹.All reagents should be in an ambient temperature when use.

².The cleaved nucleotide is the third nucleotide upstream from PAM sequence. Find gRNA target sequence as close to the stop codon as possible (TAG/CTA).

³.When isolating genomic DNA, it is preferable to remove RNAs and proteins by treatment of RNase and proteinase K before phenol-chloroform extraction.

- If homologous arms include the gRNA target sequences, divide into two PCR products, and set PCR primers inserting one nucleotide mutated on PAM sequences (NGG to NXG or NGX) (*see* Note 4). Perform PCR using a and b primer set with genomic DNA and c primer set with pX458 plasmid, and extract PCR products by electrophoresis-gel extraction (*see* Notes 5 and 6). Using extracted PCR products (a and b products) as template, perform the next PCR (a forward + b reverse primer set) having mutation on PAM sequences in the TH-Left arm. Using PCR product (TH-Left arm and c product) as template, perform PCR (a forward + c reverse primer set), and extract TH-Left arm-T2A-GFP-pA product (Fig. 1).
- c. Calculate mole number of inserting PCR product and linearized backbone plasmid. Mix the same mole amount of insert and plasmid. Add Gibson assembly reagent at the same volume of total DNA mixture, and react on 50° C for 15 min. Add ddH₂O 4 volumes of total DNA-Gibson mixture, and apply 1/10 volume to transfection into the competent cell. Confirm the insertion by PCR and sequencing.
- d. Insert hPGK-PuroR-pA and TH-Right arm as the same process with TH-Left arm-T2A-GFP-pA. After adding TH-Left arm-T2A-GFP-pA, Not I was used for linearization, and TH-Right arm was inserted (*see* Note 7). Xba I-recognition sequences were included on the forward primer of TH-Right arm to linearize TH-Left arm-T2A-GFP-pA-TH-Right arm donor template. After Xba I cut of TH-Left arm-T2A-GFP-pA-TH-Right arm donor template, hPGK-PuroR-pA was inserted (*see* Note 7). Confirm the insertions by PCR and sequencing.

3.2 Verification of Optimal gRNA

1. Purify the constructed pX458gRNA vectors using Miniprep kits according to the manufacturer's instructions.
2. 1 day before transfection, culture the HEK293FT cells (*see* Note 8) in 10% FBS media. Plate HEK293FT cells as 1×10^6 on 60 mm dishes as the number of validating pX458gRNAs.

⁴.To prevent donor template to be targeted by CRISPR-gRNA, it is preferable to add mutation on PAM sequence, called blocking mutation. When adding the mutation on PAM sequences, if the PAM sequences are on the coding region, consider not changing the amino acid. If the target sequence is not on the coding region, it is not necessary to consider [11].

⁵.Be sure to linearize plasmid by electrophoresis after gel extraction as circular plasmid is not contaminated.

⁶.When constructing donor vector, complete the electrophoresis-gel extraction after every PCR so as not to contaminate whole genome as PCR template.

⁷.Be sure that restriction enzyme recognition sequences are not included in the region previously inserted to the donor vector in every step.

⁸.It is not necessary to use HEK293 cell for optimization of gRNA. iPSCs can be used simultaneously to confirm cleavage and modify gene as dividing FACS-sorted cells.

3. Prepare 150 μL of Opti-MEM and add 11.25 μL of Lipofecta-mine 2000, and separately prepare 150 μL of Opti-MEM and add 3.75 μg of pX458gRNA. Let stand for 5 min. Then mix Lipofectamine mixture with DNA mixture, and let stand for 20 min. Add Lipofectamine-DNA mixture into the HEK293FT cells plated the previous day. Mix by moving back and forth gently. The next day, change the media.
4. Two days after the transfection, check whether the GFP is expressing (*see* Note 9). Trypsinize, neutralize, and pipette to make single cells for FACS sorting. Collect more than 2×10^5 GFP-positive cells and lysed with genomic DNA lysis buffer. Isolate genomic DNA by phenol-chloroform extraction (*see* Note 3).
5. Perform PCR using the genomic DNA from GFP-sorted cells for 40 cycles to expand the region including gRNA-targeted genomic locus. Simultaneously perform the same PCR using wild HEK293FT gDNA for control.
6. Reanneal the PCR products in a condition of 95° C 5 min, 95–85° C -2°C/s , and 85–25° C 0.1°C/s and hold at 4° C.
7. Add appropriate amount of 10XNEB2 buffer and 0.5 μL of T7E1 into the reannealed PCR product. Incubate at 37° C for 2 h.
8. Check cleaved band by gel electrophoresis (Fig. 2).

3.3 Nucleofection of Vectors into iPSCs

1. Linearize donor vector using Nco I (*see* Note 7), and purify linearized donor vector and selected pX458gRNA vector.
2. Prepare confluent iPSCs cultured on StemFlex feeder-free condition. Add 2 mL of DPBS containing calcium and magnesium with 0.5 $\mu\text{g}/\text{cm}^2$ of Laminin-521 into 60 mm dish. Incubate at 4° C overnight.
3. Aspirate the DPBS-Laminin-521 from the 60 mm dish. Add fresh StemFlex media containing 10 nM of ROCK inhibitor into Laminin-521-coated 60 mm dish, and stand by at the incubator.
4. Make iPSCs into single cells using Accutase cell detachment solution and neutralize. Count the cell number.
5. Mix 82 μL of Stem Cell Nucleofector solution with 18 μL of Nucleofector supplement. Add 5 μg of pX458THgRNA vector and 5 μg of donor vector for 2×10^6 cells.
6. Spin down 2×10^6 cells and remove the supernatant. Remove the supernatant completely using P200 pipette.
7. Add DNA mixture into the cell pellet and suspend. Transfer DNA-cell mixture into a cuvette, and apply program A-023 in Nucleofector 2b.

⁹GFP will be expressed when pX458gRNA vector is transfected because GFP is connected with SpCas9 through T2A and will gradually disappear with cell division.

8. Immediately transfer the nucleofected cells to the prewarmed Laminin-521-coated medium containing 60 mm dish [12], and incubate for 2 days (*see* Note 10).
9. One day after the nucleofection, add 2 mL of DPBS containing calcium and magnesium with 0.5 $\mu\text{g}/\text{cm}^2$ of Laminin-521 into 60 mm dish. Incubate at 4° C overnight.
10. Two days after the nucleofection, aspirate the DPBS-Laminin-521 from the 60 mm dish. Add fresh StemFlex media containing 10 nM of ROCK inhibitor into Laminin-521-coated 60 mm dish.
11. Make the nucleofected cells into single cells using Accutase cell detachment solution. Neutralize. Sort GFP-positive cells by FACS in a sanitized condition at the level of culturing.
12. Plate the GFP-positive cells on the Laminin-521-coated 60 mm dish. Incubate for 2 days for the recovery from FACS sorting.
13. Change the media every 2 days with fresh media containing 0.5 $\mu\text{g}/\text{mL}$ puromycin to select homologously recombined clones. If the cell's morphology does not look healthy after the FACS sorting, start puromycin selection several days later. Continue puromycin selection until the cells start to form colonies.
14. Select the colonies when they are enlarged enough to be selected.

3.4 Confirmation of Cloned iPSCs

After selecting appropriately enlarged colony, expand and split cells for freezing and genomic DNA isolation to verify an integration of the donor vector, i.e., either precisely integrated or randomly integrated. Use either Southern blot or genome walking PCR kit to distinguish the integrations (*see* Note 11).

3.5 Neural Differentiation from Cloned iPSCs Containing TH Reporter [5]

3.5.1 Differentiation of iPSCs Containing TH-GFP Reporter to Neural Stem Cells

1. Prepare a confluent iPSCs on 60 mm dish. At day 1, collect iPSCs by cell lifter, and spin down $200 \times g$ for 5 min.
2. Remove the supernatant and add 1 mL of NSC medium. Suspend the pellet, and spin down $200 \times g$ for 5 min.
3. Remove the supernatant and add 10 mL of NSC medium. Resuspend and plate on T25 flask. Incubate for 3 days. At day 3 the cell clumps gradually make embryoid bodies.

¹⁰. Sorting of GFP-positive cells 48 h after the nucleofection may support decreased frequency of randomly integrated clones. It is possible to skip FACS sorting and direct to puromycin selection, and pick a number of colonies.

¹¹. Set the primers: (1) on the region outside of homologous arm and (2) in the homologous arm.

4. Collect the floating cells and spin down $200\times g$ for 5 min. Remove the supernatant, and add fresh NSC medium and plate on used T25 flask. Change the media every 2–3 days.
5. At day 17, collect the floating cells and clumps, and spin down $200\times g$ for 5 min. Remove the supernatant and add 0.5 mL of Accutase cell detachment solution, and incubate for 3 min. Add 0.5 mL of trypsin neutralizer solution, and break down the cell clumps by pipetting gently using P1000 pipette. Add the used media and spin down $200\times g$ for 5 min.
6. Remove the supernatant and add 10 mL of fresh NSC media. Suspend and plate on T25 flask. Repeat 17 day process until the embryoid bodies make the neurospheres and expansion.

3.5.2 Differentiation of Dopaminergic Neuron from NSCs

1. Prepare 24-well plate and add 0.45 mL of PBS with 0.05 mL of poly-L-ornithine ($10\times$). Incubate overnight at 37°C .
2. Collect the NSCs and spin down $200\times g$ for 5 min.
3. Remove the supernatant for the next use, and add 1 mL of trypsin to the pellet. Incubate at 37°C for 5 min.
4. Neutralize with 1 mL of trypsin neutralizer, and break down the neurospheres by pipetting for 40 times. Add the media, and spin down $200\times g$ for 5 min.
5. Remove poly-L-ornithine coating and wash with PBS twice. Proceed to the next step.
6. Remove the supernatant after the centrifuge. Add fresh conditioned media, and plate on the poly-L-ornithine-coated plate.
7. Change the media every day. The neurospheres will attach on the bottom in an hour. TH-positive cells will gradually appear in 3 days (see Note 12).

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¹² TH-positive cells can be observed by immunocytochemistry and confocal imaging.

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Fig. 1.

Donor vector illustrates where it targets on TH genomic locus. T2A-GFP-pA and hPGK-PuroR-pA are integrated in place of stop codon. Blue line is gRNA target sequence, red is PAM sequence, and black head on the TH-Left arm is the mutation on PAM sequence. *a*, *b* and *c* primer sets are for the PCR to clone TH-Left arm-T2A-GFP-pA. *a* forward primer and *c* reverse primer have 16 bp sequence homologous with backbone plasmid

Fig. 2.

Illustration of T7E1 assay and example of gel electrophoresis. DSB occurs in the targeted genomic region and is repaired by NHEJ, leading Indel sequences. Reannealing of PCR product from Indel sequences and wild sequences generates homoduplex strands and heteroduplex strands. T7E1 cleaves the mismatched strands. Lane 1 is the result of gRNA#01, and lane 2 is the result of control. Upper band near 750 bp indicates uncleaved PCR products containing homoduplex of Indel and wild strands. Lower two bands indicate cleaved PCR products