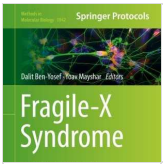

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Induced Neurons for the Study of Neurodegenerative and Neurodevelopmental Disorders

Book Chapter

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ABSTRACT

Patient-derived or genomically modified human induced pluripotent stem cells (iPSCs) offer the opportunity to study neurodevelopmental and neurodegenerative disorders. Overexpression of certain neurogenic transcription factors (TFs) in iPSCs can induce efficient differentiation into homogeneous populations of the disease-relevant neuronal cell types. Here we provide protocols for genomic manipulations of iPSCs by CRISPR/Cas9. We also introduce two methods, based on lentiviral delivery and the piggyBac transposon system, to stably integrate neurogenic TFs into human iPSCs. Furthermore, we describe the TF-mediated neuronal differentiation and maturation in combination with astrocyte cocultures.

ATTACHMENTS

Sauter2019_Protocol_InducedNeuronsForTheStudyOfNeu.pdf

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KEYWORDS

Human induced pluripotent stem cells, Nucleofection, PiggyBac transposon, Lentiviral transduction, CRISPR/Cas9, Transcription factor-mediated neuronal differentiation, Astrocyte coculture

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1 Introduction

Induced pluripotent stem cells (iPSCs) enable studying neurodevelopmental and neurodegenerative diseases such as autism spectrum disorders including fragile X syndrome and Rett syndrome, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, or spinal muscular atrophy [1]. Human iPSC lines are generated by reprogramming of fibroblasts, hair, or blood samples [2], which are either directly donated by patients with a disease relevant phenotype and a known genotype or disease-causing mutations can be introduced into the genome of the iPSCs by genomic modifications such as CRISPR/Cas9 [3]. To study the effect of the mutations on the cellular level, iPSCs can be differentiated into the disease-relevant neuronal subtypes. Conventional differentiation protocols rely on the addition of specific soluble growth factors and compounds to the culturing media. These factors trigger intracellular signaling pathways affecting transcription factors (TFs), which in turn induce neuronal differentiation by changing gene expression levels and triggering gene regulatory networks. However, these protocols can be very delicate and time-consuming, lasting from several weeks to months, and yield a heterogeneous mixture of different neuronal subtypes at different developmental stages and glia cells. The forced expression of certain neurogenic TFs in human iPSCs shortcuts neuronal differentiation resulting in rapid neurogenesis that yields highly homogeneous populations of neurons [4–7]. Here we describe the culturing of a robust inducible-neuronal iPSC line as well as different methods to introduce neurogenic TFs and genomic modifications into human iPSCs and how to differentiate those iPSCs into mature neurons.

Neurogenic TFs under the control of a doxycycline-inducible promoter can be stably integrated in the genome of iPSCs either by lentiviral delivery [8] or via the piggyBac transposon system [9]. While lentiviruses have a high efficiency in delivering transgenes, the preparation of viral particles is laborious, time-consuming and requires biosafety level 2. In contrast, the piggyBac transposon system offers a nonviral alternative to efficiently cut and paste transgenes into the genome. The production of plasmids is faster and cheaper and the piggyBac system requires only standard laboratory biosafety levels. For genome editing of human iPSCs with great precision, the CRISPR/Cas9 technology is the method of choice since it is easy-to-use, efficient, and cost-effective. Genomically modified iPSCs can be differentiated into neurons by doxycycline-induced overexpression of TFs and maturation is achieved by astrocyte coculture.

4 Notes

1. Aliquot the Matrigel according to the protocol and the dilution factor provided with it (varies for each bottle of Matrigel). We prepare aliquots for dilution in 12 ml coating medium. Briefly, thaw the Matrigel **On ice** in the cold room or the fridge and prepare a box with dry ice to precool 1.5 ml tubes. Quickly distribute the Matrigel solution into the tubes and store at **-20 °C** .
2. The piggyBac vector backbone can be obtained from Addgene (to be submitted, containing the EGFP gene

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under the control of the doxycycline-inducible promoter). For cloning of transcription factors, the EGFP can be excised using NheI and XhoI, the transcription factor cDNA can be amplified by PCR and introduced into the piggyBac vector using Gibson Assembly cloning [17].

3. There are two different lentiviral vector systems that can be used: the pLV system that consists of two constructs, one expressing the rtTA transactivator from the constitutively active EF1α promoter and the other one expressing the transgene under the control of the doxycycline-inducible TRE promoter [4] (Addgene plasmids #61472 and #61471, respectively) or the pLIX403 system that expresses the rtTA transactivator and the transgene under the TRE promoter on a single construct (Addgene plasmid #41395). The pLV plasmids that are referred to in this protocol do not contain any selection markers. If selection for the integrated constructs is required, it should be cloned into the plasmids before the production of lentiviral particles.
4. For better attachment of the neurons, freshly add **1 µl 1 mg/ml laminin solution** *per* 1 ml supplemented BrainPhys™ medium to a final concentration of **1 µg/mL** .
5. For the coculture with astrocytes, we use BrainPhys™medium with a minimal supplementation since we found that astrocytes do not grow well in the presence of cAMP. Addition of BDNF and GDNF was neither found to enhance maturation nor affect astrocytes but might be beneficial depending on experiment design.
6. Use **1 mL diluted Matrigel solution** per well of a 6-well plate, **0.5 mL** per well of a 12-well plate and **0.25 mL** per well of a 24-well plate.
7. Use **2 mL mTeSR™1 medium** per well of a 6-well plate, **1 mL** per well of a 12-well plate and **0.5 mL** per well of a 24-well plate. If you would like to avoid feeding the cells on the weekend, add at least the 1.5-fold amount of medium on Friday.
8. The optimal cell density depends on the growth rate of your iPSC line. For our cells, we seed 15,000–25,000 cells/cm^2 for maintenance of stem cells, and 30,000–50,000 cells/cm^2 for differentiation experiments.
9. Check iPSCs in 4-week intervals for mycoplasma contamination using the Universal Mycoplasma Detection Kit (ATCC® 30-1012K™) according to the manufacturer's instructions.
10. The optimal density for freezing depends on your iPSC line. For our cells, a density of 500,000–1,000,000 cells/cryotube in **0.5 mL** – **1 mL mFreSR™** works well.
11. Cells of one 100 µl nucleofection reaction can be seeded to one well of a 6-well plate or distributed to multiple wells of a 12- or 24-well plate.
12. The pulse CB-156 is recommended if higher transfection efficiency is favored at expenses of a lower survival rate, whereas the pulse CB-150 results in higher viability with lower transfection efficiency.
13. Leaving the cells in Nucleofector™ solution for extended periods of time may lead to reduced transfection efficiency and viability so it is important to work as quickly as possible. If you face problems such as low transfection efficiency due to very big plasmids etc. you can try to incubate the cells after nucleofection in the Nucleofector™solution at **Room temperature** for approximately **00:10:00** .
14. The concentration of antibiotic optimal for selection depends on the specific iPSC line of choice and should be determined with a killing curve. We use a final concentration of **20 µg/mL** for blasticidin, **3 µg/mL** for puromycin, and **250 µg/mL** for hygromycin.
15. Alternatively, the copy number can be determined as described for the lentiviral transduction (see Subheading/protocol 3.3) by performing a TaqMan®-based qPCR on genomic DNA. Use the albumin gene for normalization and a gene specific for the piggyBac construct for counting the integration events. We recommend using primers and probes for the antibiotic resistance gene, if not otherwise present in the genome of your iPSC line. It is important to have both genes present on the same plasmid used for the standard curve since the preparation of the serial dilutions is prone to small variations.
16. Before performing the copy number determination, the cells must be passaged at least once to avoid the interference of nonintegrated piggyBac plasmids with the qPCR.
17. The optimal settings of the qPCR protocol may vary with the qPCR machine and the SYBR® Green or TaqMan® mix used. The settings described in this protocol are referring to the StepOnePlus™Real-Time PCR System (Thermo Fisher Scientific), with the Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) or the TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific).
18. From this step on, the cells are producing viral particles and should be handled at biosafety level 2. All viral particles that are collected are also biosafety level 2.
19. If your centrifuge is not able to run at 7000 × g, the centrifugation step can be carried out at lower g for a longer

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period of time (e.g., at **5000 x g, 00:30:00**).

20. We usually use one aliquot of viral particles to transduce one well of a 6- or 12-well plate. In order to optimize the viral transduction, it is recommended to determine the viral titer. Therefore, transduce cells with different volumes of the virus containing supernatant and performa qPCR on genomic DNA counting the number of integrated copies per cell.
21. Directly after transfection, the iPSCs are biosafety level 2 and should be handled as such, after the medium change the next day, they are back at biosafety level 1.
22. The calculation of the molecule number per µl in a solution with a concentration of **1 mg/mL** is as follows: molecules/µl = (6.022×10^{23} 1/mol [Avogadro constant]/(length of the plasmid × 660 Da [average molecular weight of a base pair])) × 10^{-6} g/µl. For the pAlbumin plasmid with a length of 7539 bp, the calculation is: (6.022×10^{23} 1/mol/ (7539 bp × 660 Da)) × 10^{-6} g/µl = 1.2×10^{11} molecules/µl.
23. Avoid placing the PAM sequence into your sgRNA-expressing vector and the potential donor construct. It will be cut once sgRNA and Cas9 are expressed. The vectors from the Zhang lab can be ordered in different versions, that is, with GFP or puromycin expression. If positive cells should be sorted with flow cytometry, GFP is optimal. When expanding of single cells and subsequent picking of monoclonal colonies is preferred, use puromycin with the version V2 on Addgene, which is corrected from a previous version. The success rate of this cloning strategy is usually very high.
24. One or two guanines can be added for more efficiency of the U6 promoter if the designed sgRNA is not starting with it. They have to be added to the bottom oligo as reverse complement in addition to the sgRNA sequence as well.
25. The T7 endonuclease I assay is performed as follows: Transfect the sgRNA- and Cas9-expressing constructs into a test cell line (e.g., 293T/17, *see* materials and steps in protocol 3.3). Isolate the DNA using a DNA extraction kit, such as the DNeasy® Blood and Tissue Kit (Qiagen). Amplify the locus using flanking primers tested for specificity in advance. Purify the reaction using a PCR Purification kit (Qiagen). Elute in **30 µl** . Mix **200 ng purified PCR product** , **2 µl NEBuffer™ 2** and water to a total volume of 19 µl. Hybridize the PCR product in a thermocycler by heating to **95 °C** and ramp down to **85 °C** with –2 °C/s, then to **25 °C** with 0.1 °C/s and hold at **4 °C** . Add **1 µl T7 endonuclease I** to the reaction and incubate at **37 °C** for **00:20:00** . Run on a **2 % agarose gel** (**00:30:00** , 90 V) to see if one or more bands appear. If two or three bands are visible, the sgRNA works fine. The T7 endonuclease cuts at wobbles that appear with reannealing of nonfitting DNA [13] strands. This happens if the Cas9 cuts parts of the DNA of the population of cells used as the test cell line.
26. Rat Primary Cortical Astrocytes stick to the plastic used in cell culture dishes and centrifuge tubes. Prior to use, rinse all material that will come in contact with the cells with medium to prevent cells from sticking to the plastic.
27. Since the cleaning of the coverslips is very time-consuming, it can also be done in 1 day. Briefly, rinse the coverslips two times in ddH_2O, then add **50 mL 1 M HCl** and shake for **01:00:00** . Rinse three times with ddH_2O by shaking for **00:02:00** , and then rinse once more with ddH_2O by shaking for **01:00:00** . Shake three times in **100 % ethanol** for **00:02:00** and one time for **01:00:00** . Sterilize the coverslips at **225 °C** for **02:00:00** – **03:00:00** . Successful cleaning will be accompanied by an even spread of coating solution across the whole surface of the coverslip. If problems with adhesion occur, go back to the long protocol.
28. The purpose of the spacers is to allow growth of the induced neurons in close proximity to the astrocyte feeder layer but without physical contact.
29. The coverslips can have any size depending on the requirements of the experiment. We routinely use 12 mm coverslips equipped with three paraffin feet in a 24-well plate. It is recommended to add additional volume of medium to the well to completely cover the coverslips in order to avoid floating.
30. We add approximately **50 µl ddH2O** per week for a 24-well plate to compensate for volume loss due to evaporation. Store a test plate full of H_2O in the incubator and weigh in weekly intervals to check for evaporation.

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ABSTRACT

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FILES

3.1 Human iPSC Culture
Version 1
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3.2 Nucleofection of iPSCs
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3.3 Lentivirus Production and Transduction
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3.4 Genome Editing with CRISPR/Cas9
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3.5 Doxycycline-Induced Differentiation
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3.6 Coculturing with Astrocytes
Version 1
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