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Genetic Engineering of Human Pluripotent Stem Cells using PiggyBac Transposon System:

hPSC engineering with PiggyBac transposons

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Abstract

Human pluripotent stem cells (hPSCs) emerged as an important tool to investigate human development and diseases. These studies often require genetically engineering hPSCs to stably express a transgene, which remains functional in various hPSC progeny. PiggyBac transposon is a highly effective and technically simple vector system with large cargo space available for permanent gene delivery. This unit describes the use of PiggyBac transposons for genetic engineering of hPSCs to introduce conditionally expressed transgene or reporter to effectively monitor gene expression during differentiation. Both methods enable robust generation of stable hPSC lines within one month.

Keywords

genetic engineering; PiggyBac transposon system; human pluripotent stem cells

INTRODUCTION

Genetic engineering of human pluripotent stem cells (hPSCs) offers a powerful and versatile tool to study gene function in human development and diseases. Introducing exogenous conditionally expressed genes or gene reporters is commonly used to investigate gene function in hPSC system. There are several gene delivery systems available. Random integration is a traditional approach to insert exogenous genes using plasmid. However, transfection of hPSCs with plasmid is typically a very low efficiency and poses the risk of endogenous gene disruption due to random plasmid integration. Retroviral or lentiviral-based vectors offer a more effective tool for gene expression in hPSCs. However, potential transgene integration into the promoter/enhancer or gene coding region, a limited cargo

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capacity (Astrid et al., 2002; Rick et al., 2004; Rong et al., 1996) along with transgene silencing (Ellis, 2005) limit the utility of retro- and lentiviral vectors. In addition, lentivirus production is laborious and challenging, particularly when high titer of virus is required. Recently, researchers found so-called safe harbor loci available in the genome, which allows for site-specific transgene integration and consistent gene expression without gene disruption. Among these safe harbor loci are adeno-associated virus site 1 (AAVS1), the chemokine (C-C motif) receptor 5 (CCR5), ROSA 26 locus and ENZY locus (Costa et al., 2005; DeKaveler et al., 2010; Hockemeyer et al., 2009; Lombardo et al., 2011; Sadelain, Papapetrou, & Bushman, 2012). However, a single harbor locus only allows for integration of two copies of transgene. Due to this limitation, transgene expression from safe harbor loci in hPSCs is often weak and prone to silencing following differentiation.

The PiggyBac system became a promising tool for non-viral genetic engineering of hPSCs. This system consists of PiggyBac transposon vector and transposase vector, which transiently expresses transposase enzyme. PiggyBac transposon is a mobile genetic element that efficiently inserts copy of DNA into the genome through a “cut-and-paste” mechanism. PiggyBac transposition requires transposase enzyme that recognizes transposon specific inverted terminal repeats (ITRs) and efficiently integrates the transposon vector into TTAA chromosome sites randomly scattered throughout the genome (Woodard & Wilson, 2015; Yusa, Zhou, Li, Bradley, & Craig, 2011). Mapping PiggyBac integration sites in human cells has found that 97% of integrations occurs within introns (Ding et al., 2005; Rostovskaya et al., 2012; Wilson, Coates, & George, 2007). Other studies revealed that the PiggyBac transposase has an insertional preference into the transcriptional control regions (Huang et al., 2010; Yoshida et al., 2017). The transposase expression vector can be co-transfected with one or more PiggyBac transposon vectors to achieve simultaneous expression of multiple transgenes (Woodard & Wilson, 2015). PiggyBac system allows for tight control of the number of transgene integration by adjusting the transposase/transposon ratio (Burnight et al., 2012; Wang et al., 2008; Wilson et al., 2007). Another unique advantage of PiggyBac, is that a donor cassette can be excised using excision-only PiggyBac transposase in a scarless fashion. This approach demonstrated its utility for generating transgene free induced pluripotent stem cells (iPSCs) (Woltjen et al., 2009). Importantly, PiggyBac system has the large cargo capacity suitable for delivery of up to several hundred kilobases of DNA (Li et al., 2011; Rostovskaya et al., 2012). In our hands, we achieved a good efficacy of transgene delivery into hPSCs using PiggyBac with cassette size of up to 10kb. We also found that drug-inducible genes expressed in hPSCs via PiggyBac under control of EF1a promoter and TRE, are not silenced during differentiation and can be conditionally upregulated at the desired developmental stages.

Here, we describe two protocols for genetically engineering hPSCs for most typical experimental applications using PiggyBac transposons. Basic Protocol 1 describes the generation of hPSCs with drug-inducible gene expression using mTeSR1 and subsequent selection of genetically modified cell lines by culture of transfected cells at low density on Matrigel. Basic Protocol 2 describes the generation of reporter cell lines to monitor WNT/ β -catenin activity throughout differentiation. This protocol uses chemically-defined E8 media and culture of FACS-sorted single cells on Vitronectin to select genetically engineered hPSC lines. These protocols are applicable to any hPSC line, including human embryonic stem

cells (hESCs) and induced pluripotent stem cells (hiPSCs), which are available from multiple cell sources including WiCell (Madison, WI, USA).

BASIC PROTOCOL 1

GENERATION OF HPSC LINES WITH CONDITIONAL GENE EXPRESSION

In this protocol as an example for conditional gene expression, an ETS1 gene under tetracycline responsive element (TRE) promoter along with M2rtTA (reverse tetracycline transactivator) will be introduced into hPSCs using PiggyBac transposons as an example. For this purpose, we will use two transposon vectors. In one vector ETS1 downstream of TREtight promoter along with Zeocin resistance gene driven by the EF1 α promoter, are subcloned between ends of two ITRs of the transposone vector. ETS1 is linked with Venus through a 2A self-cleaving peptide sequence to allow for easy monitoring of transgene expression following addition of doxycycline to cell cultures. In another vector, M2rtTA promoter linked with Puromycin resistance gene through 2A peptide sequence, is subcloned between ends of two ITRs (Figure 1). The use of two different antibiotic-resistance genes allows for the selection of clones that incorporate the two vectors in a single step. Although all transgene sequences for conditional gene expression could be potentially cloned into a single plasmid, a two plasmid system provides more flexibility and permits the adjustment of the TRE/M2rtTA ratio to optimize hPSC generation with robust DOX-dependent gene expression, while avoiding transgene leakage. In this protocol, hPSCs are transfected and cultured in mTeSR1 medium on Matrigel. Selection of homogenous cell populations can be achieved by picking up individual hPSC colonies from low density cultures. We have found that this protocol can be applied for conditional expression of various genes, including *ETV2*, *GATA2*, *FLI1*, and *SOX17*.

Materials

Human pluripotent stem cells (*e.g.* human embryonic stem cell lines H1, WA01 (WiCell Research Institute))

mTeSR1 defined feeder free medium (StemCell Technologies, cat. no. 05850)

Matrigel human ES cells qualified matrix (Corning, cat. no. 354277)

10 mM Y-27632 ROCK inhibitor (Tocris Bioscience, cat. no. 44230, see recipe)

0.5 mM EDTA (see recipe)

1 $\times$ TrypLE (see recipe)

Endotoxin free PiggyBac plasmid containing ETS1 ORF DNA linked with Venus under control of TREtight promoter and Zeocin resistance gene (PBTRE-ETS1 vector customized from empty backbone vector, Transposagen, cat. no. SPB-007), Figure 1A.

Endotoxin free PiggyBac plasmid DNA expressing M2rtTA (PBM2rtTA vector, customized from empty vector backbone, Transposagen, cat. no. SPB-007)

Endotoxin free plasmid DNA expressing Super PiggyBac transposase (sPBo, Transposagen, cat. no. SPB-DNA)

Nucleofector 2b device (Lonza, cat. no. AAB-1001)

Human Stem Cell Nucleofector Kit 2 (Lonza, cat. no. VPH-5022)

Zeocin (Thermo Fisher, cat. no. R25001)

Puromycin (Thermo Fisher, cat. no. A1113803)

Doxycycline (MP Biomedicals LLC, cat. no. 198955, see recipe)

Hausser Bright-Line Phase Hemocytometer

Objective marker (Nikon, cat. no. MBW10000)

Sterile 6 well tissue culture plates

Sterile 12 well tissue culture plates

Sterile 1.5 ml microcentrifuge tube

Sterile conical tubes

Humidified 37°C incubator with 5% CO₂

MACSQuant Analyzer (Miltenyi Biotech)

Prepare reagents and cells

1. Expand hPSCs under feeder free conditions in mTeSR1 medium on 6 well tissue culture plate coated with Matrigel.

Matrigel is temperature sensitive and should be kept on ice during handling. It is best to avoid freeze thaw cycles and prepare ~120 µl aliquots for one 6 well plate and store at −20°C. Specific aliquot volumes vary by lot. Each aliquot should be quickly dissolved in 12 ml cold PBS and subsequently add 2 ml to each well of a 6 well plate. Incubate at least 1 hr at room temperature or store overnight at 4°C until ready to use. Matrigel coated plates can be kept at 4°C for several weeks.

2. When cells reach ~70% to 80% confluence, add 1 ml TrypLE per well and incubate at room temperature for 2-5 min to allow detachment.

Cell confluence on the day of transfection is important for viability. Avoid using over confluent cells and treating with TrypLE greater than 5 min. Monitor optimal TrypLE treatment time under the microscope. Avoid overexposure cells to TrypLE. After cell detachment, immediately move to step 3.

3. Add 1 ml mTeSR1 medium and transfer cells to a sterile conical tube and centrifuge for 5 min at 300 × g at room temperature. Carefully remove and discard the supernatant.
4. Resuspend cells in 10 ml mTeSR1 medium. Pipet gently to achieve a single cell suspension. Remove a sample of cells for counting.
5. For each nucleofection reaction, prepare the following DNA mixture:

10 µg PBTRE-ETS1 (transposon) plasmid

3 µg PBM2rtTA (transposon) plasmid

2 µg sPBo (transposase) plasmid

100 µl transfection solution

Prepare high quality and concentrated plasmid DNA to achieve optimal transfection efficiency. It is recommended to use a 1:5-1:10 transposase/transposon vector ratio for transfections. Higher ratio may cause transgene leakage. The ratio PBTRE and PBM2rtTA plasmid in reaction mixture may require adjustment depending on gene of interest and cell type. In our hands 3:1 PBTRE/PBM2rtTA ratio is optimal. Too high PBTRE/PBM2rtTA ratio may lead to transgene leakage in hPSCs, while too low ratio may prevent strong gene expression following DOX treatment. We also noted that M2rtTA expressed at high level can cause spontaneous differentiation.

Transfect cells

6. For each reaction, transfer the required number of cells (1×10^6 cells) to a new conical tube and centrifuge 5 min at $300 \times g$.
 7. Aspirate off most of the medium and then use a pipet to remove the final layer of medium without disturbing the cell pellet.
 8. Resuspend pellet in 100 µl Nucleofector solution per reaction
 9. Promptly combine 15 µg DNA mixture with the 100 µl of resuspended cells, and transfer into a Nucleocuvette.
 10. Place Nucleocuvette into the Nucleofector device and run program B16.
- The Lonza Nucleofector 2b device comes with several preset programs for hPSCs. Each program produces different transfection efficiency and cell viability. It is recommended to find the optimal program for each specific experiment.
11. Add 500 µl mTeSR1 medium with 10 µM ROCK inhibitor to each nucleocuvette.
 12. Transfer cells to a new sterile conical tube and add 12 ml mTeSR1 medium with 10 µM ROCK inhibitor. Resuspend and transfer 2 ml of cells into each well of a Matrigel coated 6 well plate and place in the incubator.

The use of ROCK inhibitor is highly recommended to enhance single cell survival post nucleofection. Plating cell density is important for picking colonies and cell viability. If cells are plated at a high density, picking individual colonies will be difficult, but if plated cell density is too low, their survival will be compromised.

13. Change mTeSR1 medium without ROCK inhibitor at 24 hr post transfection.
- Plating hPSCs in mTeSR1 medium with ROCK inhibitor changes their morphology. However, morphology should return in mTeSR1 medium without ROCK inhibitor within 2-3 days.

Pick colonies and establish cell lines

14. Change mTeSR1 medium daily for 3-4 days without ROCK inhibitor and selection antibiotics.

15. Change fresh mTeSR1 medium with 0.5-1 µg/ml Zeocin and 0.5-1 µg/ml Puromycin daily for 10-15 days.

Should wait 3-4 days post transfection before treating with selection antibiotics to allow for cell recovery. PBTR-ETS1 plasmid contains Zeocin resistance gene and PBM2rtTA plasmid contains Puromycin resistance gene.

16. Prepare a 12 well tissue culture plate coated with Matrigel for colony transfer, aspirate the supernatant from Matrigel coated plate and add 1 ml mTeSR1 medium with 10 µM ROCK inhibitor to each well.

17. Mark colonies in antibiotic selection cultures with Nikon objector marker.

18. Check marked colonies under fluorescence microscopy and exclude those with leaky expression.

PBTR-ETS1 plasmid contains Venus expression region linked with ETS1 for monitoring gene expression. Venus protein should not be expressed without Doxycycline treatment. If some PiggyBac DNA integrates into highly open chromatin regions, Venus protein could be expressed without Doxycycline.

19. Pick marked colonies using a 200 µl pipette tip and transfer into a sterile 1.5 ml microcentrifuge tube.

20. Pipet up and down to break apart the colony.

21. Transfer the colony into mTeSR1 medium with 10 µM ROCK inhibitor onto each well coated with Matrigel of a 12 well plate.

It is important to use mTeSR1 medium with ROCK inhibitor. Otherwise, most cells will die post colony picking. High plating density post colony picking is important for cell survival. A 24 or 12 well plate is optimal for hPSC culture from one picked single colony.

22. Change mTeSR1 medium without ROCK inhibitor at 24 hr post colony picking.

23. Change fresh mTeSR1 medium until cells reach ~70% to 80% confluence.

24. For passaging, aspirate cell culture medium and add 1 ml EDTA per well to hPSCs. Incubate at room temperature for 3-5 min to allow detachment.

25. Aspirate EDTA solution carefully and add 2 ml mTeSR1 medium. Transfer cells to a new sterile conical tube.

26. To prepare 6 well tissue culture plate coated with Matrigel, aspirate the supernatant from Matrigel coated plate and add 1 ml mTeSR1 medium to each well.

27. Transfer cells from one conical tube to 2 wells of a 6 well plate coated with Matrigel.

One well is for monitoring Doxycycline treatment and the other is for freezing stock.

28. Treat with 1-2 µg/ml Doxycycline.

29. Monitor the cells after 24-48 hr under fluorescence microscopy and choose colonies with homogeneous Venus expression.

Depending on integration sites, some colonies may exhibit gene silencing effect. The other colonies may show heterogeneous gene expression.

30. Confirm DOX-dependent transgene expression by RT-PCR.

31. Freeze cells without Doxycycline.

Inducible transgene expressing cells should be maintained without Doxycycline, the cells will lose pluripotency with continued Doxycycline treatment because ETS genes affect pluripotency. Monitoring and freezing cell stocks should be different batches. Doxycycline treated cells should not be used as freezing stock.

BASIC PROTOCOL 2

ESTABLISHMENT OF REPORTER hPSC LINES

In this protocol for generating reporter hPSC lines, TOP-EGFP PiggyBac vector that contains 7 copies of the wild type TCF/LEF-binding region is introduced into hPSCs (Figure 2A). When WNT signaling is activated, β -catenin translocates into the nucleus and binds to TCF, leading to EGFP expression (Figure 2B and C). In addition, this vector contains antibiotic resistance gene Zeocin driven by EF1 α promoter (Figure 2A). This protocol uses hPSC culture in chemically defined E8 medium on Vitronectin XFTM, and single cell sorting for clonal hPSC isolation. In this example, substances that activate WNT/ β -catenin signaling such as LiCl may cause high background reporter activity in transfected cells. Since TeSR1 medium contains LiCl, it should be avoided for this application. Following establishment of TOP-EGFP reporter hPSC lines, the same protocol can be used to generate FOP-EGFP cell lines for use as a negative control. FOP DNA has 7 copies of mutated TCF/LEF binding sites which prevents activation of EGFP expression in presence of intranuclear β -catenin.

Materials

Human embryonic stem cell line H1, WA01 (WiCell Research Institute)

TeSR-E8 media (StemCell Technologies, cat. no. 05990)

Vitronectin XFTM (StemCell Technologies, cat. no. 07180)

10 mM Y-27632 ROCK inhibitor (Tocris Bioscience, cat. no. 44230, see recipe)

10 mM CHIR99021 (Tocris Bioscience, cat. no. 4423, see recipe)

0.4% Trypan Blue (Thermo Fisher cat. no. 15250061)

0.5 mM EDTA (see recipe)

1 $\times$ TrypLE (see recipe)

Endotoxin free plasmid PiggyBac DNA expressing TOP-EGFP (PBTOP-EGFP, customized from empty vector backbone Transposagen vector, cat. no. SPB-007), Figure 2A.

Endotoxin free plasmid DNA expressing Super PiggyBac transposase (sPBo, Transposagen, cat. no. SPB-DNA)

Nucleofector 2b device (Lonza, cat. no. AAB-1001)

Human Stem Cell Nucleofector Kit 2 (Lonza, cat. no. VPH-5022)

Zeocin (Thermo Fisher, cat. no. R25001)

Hausser Bright-Line Phase Hemocytometer

Sterile 6 well tissue culture plates

Sterile 96 well tissue culture plates

Sterile 1.5 ml microcentrifuge tube

Sterile conical tubes

Humidified 37°C incubator with 5% CO₂

FACSAria II (BD)

Prepare and transfect cells

1. Expand hPSCs in feeder-free system in TeSR-E8 medium on tissue culture plates coated with Vitronectin XFTM.

Vitronectin XFTM is not temperature sensitive and can be thawed at room temperature. Dilute Vitronectin XFTM in 1× PBS to reach a final concentration of 10 µg/ml (2 ml of Vitronectin XFTM/50 ml 1× PBS) and gently mix by pipetting. Coat 1 ml/1 well for 6 well plate with diluted Vitronectin XFTM. Incubate at room temperature for at least 1 hr before use. If it is not used immediately, the culture plate must be sealed and stored at 4 °C for up to 1 week after coating. Stored coated culture plates needs to be brought to room temperature 30 min before cells are passaged.

2. Change fresh TeSR-E8 medium daily.
3. When cells reach ~70% to 80% confluence they are ready for transfection.

When cells reach 80% confluency, cell number would be $2-3 \times 10^6$ /1 well for 6 well plate. If cells are cultured over confluent or for more than 5 days, cells will spontaneously differentiate with decreased transfection efficiency after subculture.

4. For nucleofection, prepare the following DNA mixture:

5 µg TOP-EGFP (transposon) plasmid

0.5 µg sPBo (transposase) plasmid

100 µl transfection solution

It is recommended for DNA plasmids to use pure, concentrated, and endotoxin-free plasmid DNA. DNA volume must not exceed 10% of the nucleofection reaction; high amounts of DNA affect cell viability. To increase rates of DNA integration, amounts of DNA can be adjusted. However, if amount of transposase plasmid is greater than 5 µg, excessive DNA integration may occur causing spontaneous differentiation and loss of pluripotency.

5. Aspirate medium from culture plate and wash hPSCs with 1 ml PBS.
6. Add 1ml TrypLE and incubate for 2-5 min at 37°C until cells have detached from the wells.

When cells are incubated with TrypLE solution, do not incubate over 5 min. If cells are incubated with TrypLE solution longer than 5 min, they aggregate and are difficult to resuspend following centrifugation.
7. Resuspend the cells in 9 ml TeSR-E8 medium and transfer to a 15 ml conical tube and centrifuge 5 min at $300 \times g$, at RT.
8. Carefully remove supernatant and resuspend in 2 ml TeSR-E8 medium.
9. Mix 10 µl of 0.4% trypan blue and 10 µl medium with cells, and count cell numbers.
10. For transfection, transfer the required number of hPSCs (1×10^6 cells) to a new 15 ml conical tube, fill with 10 ml TeSR-E8 medium and centrifuge 5 min at $300 \times g$, at RT.

If different numbers of cells are used in the transfection reaction, DNA amounts should be adjusted proportionally to cell numbers.
11. Aspirate supernatant and remove extra media by pipetting without touching cell pellet.
12. Resuspend pellet with DNA and nucleofection solution and transfer to Nucleocuvette.

If cell medium is not completely removed it will lead to poor transfection efficiency. Cytokines and medium reagents inhibit transfection efficiency.
13. Insert the cuvette with cell mixture into Nucleofector device and run program A13.
14. Transfer cell mixture into 15 ml conical tube, then fill with 12 ml TeSR-E8 medium with 12 µl ROCK inhibitor (10 µM) and resuspend cells by pipetting once or twice.
15. Aspirate the Vitronectin XF™ solution from coated 6 well plate and transfer transfected cells to each well and place in the incubator.

For increased cell viability, plate at a high density. Recommended plating cell density is $4-6.5 \times 10^5/\text{cm}^2$.

16. After 24 hr post transfection, change media with TeSR-E8 media without ROCK inhibitor. Change the medium to fresh TeSR-E8 medium daily.
Antibiotic treatment should be avoided during first 3-4 days post-transfection.
17. After 3 or 4 days, add 0.5-1 µg/ml of Zeocin and culture for an additional 2 days.
After 3 or 4 days, DNA should be integrated into genome allowing for transfected cells selection with antibiotics. Prior to antibiotic treatment, the optimal antibiotic concentration must be established using non-transfected cells. Sensitivity to antibiotic treatment is variable among different hPSC lines.
18. Collect cells using 1× TrypLE and transfer into a new Vitronectin XF™ coated plate with TeSR-E8 medium containing Zeocin and ROCK inhibitor and culture for an additional 5 days.
One-time subculture is essential to ensure cell viability following cell sorting in next step.

Clone transfected hPSCs by single cell sorting

19. Before sorting, coat 96 well plates with Vitronectin XF™ for 1 hr at room temperature.
If using 96 well plates that were already coated with Vitronectin XF™ and stored at 4C, plate must incubate at least 30 min at room temperature before sorting to increase viability of sorted cells.
20. Aspirate Vitronectin XF™ solution from 96 well plate and add 200 µl TeSR-E8 medium with 10 µM ROCK inhibitor. Before sorting, keep 96 well plates at 37°C.
21. Aspirate media from the culture plate in step 18 and wash transfected hPSCs with 1ml PBS.
22. Add 1ml 1× TrypLE and incubate for 2-5 min at 37°C until cells have detached.
23. Resuspend cells in 9 ml TeSR-E8 medium and transfer to 15 ml conical tube and centrifuge 5 min at 300 × g at room temperature.
24. Carefully remove supernatant and resuspend cells in 2 ml medium with 10 µM ROCK inhibitor.
25. Filter suspension through nylon mesh or cell strainers (40 µm) into FACS tubes. Keep cells on ice.
26. Using FACS Aria, single cell sort to 96 well plates.
Cells are first gated by FSC-A vs. SSC-A and second gated by both FSC-A vs FSC-H and SSC-A vs SSC-H for removing all doublets. To exclude leaky cells from sorting, use only the EGFP-negative population for sorting. Sorting speed should be less than 2000 cellular events/second to decrease potential sorting errors.
27. After sorting, incubate plates at 37°C.
28. After 2 or 3 days, change to 200 µl TeSR-E8 medium without ROCK inhibitor.

Wait to change fresh medium until 2-3 days post sorting because it is easy to lose cells from the plate. Small colonies composed of 3 or 5 cells should be visible in the well after 2-3 days.

29. Change medium every 2 days until cells expand and generate a colony for passaging (around 8-10 days).

30. Collect the colony by treating cultures with 0.5 mM EDTA for 2 min, after then aspirated EDTA solution and resuspend cells in 200 μ l of TeSR-E8 medium by pipetting. Transfer cells to 24 well plates coated with Vitronectin XFTM.

Expand and select the proper clones

31. Expand cell clones by passaging in TeSR-E8 medium on Vitronectin XFTM.

During media changes, the medium should not be aspirated completely to prevent cell drying. During expansion, monitor cell lines under fluorescence microscopy and eliminate leaky cells. Although EGFP-positive cells are excluded by FACS, some leaky colonies (1 or 2%) still can still be found.

32. After expanding transgenic hPSC lines, test reporter functionality of reporter by feeding cells with fresh TeSR-E8 medium containing 4 μ M CHIR99021.

33. Keep plate at 37°C for 24 hr.

34. Change to fresh TeSR-E8 medium from plate and check the cells under fluorescence microscopy or by flow cytometry.

35. Select clones that lack EGFP expression in TeSR-E8 medium without CHIR99021, but upregulate EGFP expression following CHIR addition.

Depending on integration sites, some clones may show reporter silencing effect with CHIR99021.

REAGENTS AND SOLUTIONS

For culture recipes, use sterile tissue culture-grade water.

Y-27632 ROCK inhibitor, 10mM—Dissolve Y-27632 Rho-associated protein kinase inhibitor (DeKolver et al.) to 10mM in ddH₂O. Aliquot and store for up to 1 year at -20°C.

EDTA, 0.5 mM—Resuspend 500 μ l 0.5 M EDTA in 499.5 ml 1X PBS without calcium and magnesium (Sigma) and sterilize by passing through a 0.22 μ m filter store at 4°C.

1X TrypLE—Resuspend 50 ml 10 $\times$ TrypLE select (Thermo Fisher, cat. no. A1217702) and 1 ml 0.5 M EDTA in 449 ml 1X PBS without calcium and magnesium (Sigma) and sterilize by passing through a 0.22 μ m filter and store at 4°C.

Doxycycline, 1 mg/ml—Dissolve Doxycycline to 1 mg/ml in ddH₂O. Aliquot in an amber microcentrifuge tube for light protection and store at -20°C.

CHIR99021, 10 mM—Resuspend 1 mg of CHIR99021 in 210 μ l DMSO. After reconstitution, solution is aliquoted and stored in tightly sealed tubes and stored at -20°C and used within 1 month.

COMMENTARY

Background Information

Genetic engineering of hPSCs offers an important tool to assess gene function in development and diseases. Although multiple gene editing technologies became available for hPSC modification, the simplicity and large cargo space available with the PiggyBac system makes it an attractive tool for rapid and efficient generation of reporter hPSC lines, or lines with conditional gene expression. The PiggyBac method requires a simple hPSC transfection without laborious virus production and makes it possible to generate a desired hPSC line within one month.

Critical Parameters

The most critical steps for successfully engineering hPSC lines using PiggyBac transposons are: 1) the use of high quality hPSC lines; 2) efficient hPSC transfection with PiggyBac vectors; 3) optimizing the amount of transfected DNA and transposon/transposase ratio; 4) optimizing antibiotics dosages (Okita, Ichisaka, & Yamanaka, 2007) for selection of transduced clones and the conditions for post-transfection cultures.

A homogenous population of good quality hPSCs are required for these protocols. Cells should exhibit normal PSC morphology and express pluripotency markers SSEA4, OCT4, SOX2, NANOG and Tra-1-60. hPSC cultures with spontaneous differentiation should be avoided. hPSCs should be passaged 2-4 days before transfection to make sure that cells are actively proliferating before being utilized. After genetic modification, pluripotency of selected hPSC lines should be confirmed again by teratoma assay and assessing expression of SSEA4, OCT4, NANOG, SOX2 and Tra-1-60 pluripotency markers. Cytogenetic analysis should be performed to ensure preservation of normal karyotype.

Electroporation is the most efficient method for gene transfer to hPSCs. Typically, transfection efficiency by electroporation in hPSCs is up to 90%, which is much higher than transfection efficiency with cationic lipid (typically around 40%). However, transfection efficiency depends on plasmid size. The delivery efficacy for large (6-12kb) plasmids is much lower. We recommend performing pilot experiments to optimize the transfection conditions for each hPSC line to achieve high gene delivery, while minimizing cell death. This can be done using EGFP expressing plasmid with size comparable to the PiggyBac transposon vector intended to use for genetic modification. We typically use the A13 or B16 program and Human Stem Cell Kit Number 2 for PiggyBac transfection with the Lonza Nucleofector. We found that this combination works well for H1 hESCs and various iPSC lines. However, other options for hPSC transfection are available and may improve transfection efficacy of different cell lines. For example, Lonza recommends specific Nucleofector kits for improving transfection efficiency of H9 hESCs.

To achieve high transfection efficiency, it is essential to use a well-prepared single cell suspension. Presence of cell clumps, prolonged exposure to TrypLE, or excessive pipetting, reduces transfection efficacy and increases cell death. It is important to use highly pure endotoxin free DNA for transfection. The fraction of DNA solution in the nucleofection reaction should not exceed 10%, and DNA concentration in transfection solution should not exceed 40 µg per reaction. High DNA and DNA buffer content in the transfection solution can cause cell death due to toxicity. In addition, high DNA concentration can cause excessive integration, which could lead to spontaneous differentiation or transgene leakage. The use of over-confluent hPSC cultures, which is more difficult to transfect, should be avoided. Also, mycoplasma contamination is a common reason for poor or non-reproducible transfection. Check regularly cell cultures for mycoplasma contamination.

The PiggyBac system can be tightly controlled gene expression levels by the transposase to transposon ratio (Burnight et al., 2012; Wang et al., 2008; Wilson et al., 2007). A high ratio may lead to excessive gene incorporation into the genome and transgene leakage, while a low ratio may lead to weak transgene expression and silencing following differentiation. In this protocol, we recommend a 1:5-1:10 ratio of transposase to transposon vector in hPSCs. However, it depends on cell lines, transposon vector size and transfection methods, and needs to be optimized for individual conditions.

Optimal antibiotic concentration and cell density are critical to achieve effective selection of transfected cells. In our hands, 0.5-1 µg/ml concentration of puromycin or zeocin is optimal for selection. However, since hPSC lines have different growth characteristics, their sensitivity to antibiotic treatment is variable. Thus, before transfection, sensitivity of wild type hPSCs to a particular antibiotic should be established using a titration curve to select the lowest effective dose. Following transfection, we plate cells at a low density, $2-4 \times 10^4/\text{cm}^2$. Since hPSCs are motile and better survive in the presence of cell:cell contact (Barbaric et al., 2014), plating cells at higher densities increases frequencies of mixed colony formation and reduces the chances of truly clonal hPSC line generation. If a single cell sorting protocol is used, hPSCs can be plated in cultures with antibiotics at higher cell densities, e.g. $4-6.5 \times 10^5/\text{cm}^2$, to increase cell survival, because in this protocol, clonal hPSC lines will be established from already antibiotic-selected cells by single cell sorting. When cells are prepared for single cell cloning by FACS, it is important to pass cell preparations through a 40 µm cell strainer to remove cell clumps and debris. For sorting, cells should be prepared in FACS buffer without sodium azide (1X PBS, with 5% FBS and 2 mM EDTA) and kept on ice for no more than 2 hr. Most media contain phenol red, which increases background fluorescence especially to 488 nm (blue) laser line. The use of a 85 µm nozzle during sorting to decrease sheath pressure, along with a sub-2000 cellular events per second sorting speed help to accomplish the accurate single cell deposition and avoid excessive cell death. It is critical to use fresh ESC medium. Since E8 and mTeSR1 contains insulin, FGF and TGFβ, repeated medium warming causes the degradation and loss of activity of these reagents. After sorting, try to avoid media changes before small 2-3 cell aggregates become visible under microscope (typically day 2-3 post-sorting) to avoid incidental cell aspiration and lose cells from plate.

Transgene functionality in established hPSC lines should be confirmed using doxycycline treatment to prove conditional gene expression, or by using small molecules and cytokines to activate reporter function. It is also important to assess transgene functionality at different stages of differentiation because transgene silencing following differentiation can occur. Increasing transgene delivery by adjusting the transposone/transposase ratio may help to avoid transgene silencing in hPSC progeny. For conditional gene expression, it is important to select the optimal doxycycline concentration. In our experience, 1-2 µg/ml is optimal doxycycline concentrations for the induction of transgene expression in hPSC lines engineered with PiggyBac transposons. Doxycycline concentration below 1 µg/ml is typically ineffective in inducing gene expression, while high doxycycline concentration (above 2µg/ml) may cause cytotoxicity. The optimal doxycycline concentration is also depended on the gene used for overexpression and stage of differentiation.

Understanding results

Using our protocols, one can expect to achieve PiggyBac transposon transfection efficiencies around 60-80%, if proper attention is made to optimization of conditions for hPSC transfection. At this transfection efficacy the number of antibiotic resistant colonies vary from 50 to more than 300 per 10⁶ electroporated hPSCs. Typically, screening 20-30 colonies for transgene functionality is sufficient to select 2-3 good cell lines.

Time considerations

When DNA and hPSCs are ready, transfection of hPSCs with plasmid takes less than 1 hr. Three to four days post transfection, cells should be treated with antibiotics for 10-15 days. After selection of antibiotic resistant colonies, individual hPSC colonies can be picked. The expansion and assessment of transgene functionality requires further 2 weeks of further culturing. If cell cultures are collected and single cell sorted into 96 well plates, it takes at least over 7-10 days to establish a colony from a single cell. Expansion of single cell-derived colonies by passaging into a larger 24, and subsequently 6 well, plate followed by the assessment of transgene functionality can take another 2 weeks.

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Figure 1.
(A) PiggyBac transposon vectors designed for establishing of conditional ETS1-hPSC line. The TRE construct is designed to drive tetracycline-regulated expression of target gene (ETS1) and Venus gene, a bright-yellow variant of Green Fluorescent Protein. ETS1 gene and Venus gene are linked by 2A self-cleaving sequences to co-express both genes. The TRE construct also includes EF1 α promoter that express Zeocin gene, drug selection marker. BGH and SV40derived sequences drive mRNA splicing and polyadenylation. The M2rtTA construct includes EF1 α promoter to express M2rtTA gene, reverse tetracycline-controlled

transactivator, and Puromycin gene, drug selection marker. M2rtTA gene and Puromycin gene are linked by 2A self-cleaving sequences. SV40 derived sequences downstream of the M2rtTA-2A-Puro drive mRNA splicing and polyadenylation. The constructs have ITR recognition sites and core insulators at both ends of insert. TRE, Tet Response Element promoter; ETS1, ETS Proto-Oncogene 1; 2A, 2A self-cleavage peptide; Venus, Venus fluorescent protein; EF1 α , Elogation Factor 1 alpha promoter; M2rtTA, reverse tetracycline-controlled transactivator; Zeo, Zeocin; Puro, Puromycin; ITR, Inverted Terminal Repeats; BGHpA, BGH Poly A; SV40 pA, SV40 PolyA. (B) and (C) Fluorescent and phase contrast images of conditional ETS1 hPSCs line cultured with or without 2 μ g/ml Doxycycline for 2 days. (D)-(F) Dot plots showing Venus expression in wild type H1 hESCs, ETS1-hPSCs in cultures without Doxycycline and in cultures treated with 2 μ g/ml Doxycycline for 2 days. (G) Confirmation of Doxycycline-dependent ETS1 transgene expression by qPCR.

Figure 2.

(A) PiggyBac transposon vector designed for establishing WNT/β-catenin reporter TOP-hPSC line. TOP consists of 7 copies wild TCF/LEF binding sites fused with a minimal TA viral promoter driving EGFP expression. BGH derived sequences downstream of the EGFP drive mRNA splicing and polyadenylation. This construct includes EF1α promoter that express Zeocin gene, drug selection marker. SV40 derived sequences downstream of the Zeocin drive mRNA splicing and polyadenylation. This construct has ITR recognition sites and two core insulators flanking the TOP-EGFP/EF1α promoter-Zeocin. TOP, Wild TCF/LEF binding site; EF1α, Elevation Factor 1 alpha promoter; EGFP, Enhanced Green Fluorescent Protein; Zeo, Zeocin; ITR, Inverted Terminal Repeats; BGHpA, BGH Poly A; SV40 pA, SV40 PolyA. (B) Fluorescence image and phase contrast images of TOP-hPSCs in cultures without CHIR99021. (C) Fluorescence image and phase contrast images of TOP-hPSCs treated 4 μM CHIR99021 for 1 day to activate WNT/β-catenin signaling.