Video Article

Transfection, Selection, and Colony-picking of Human Induced Pluripotent Stem Cells TALEN-targeted with a GFP Gene into the AAVS1 Safe Harbor

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Abstract

Targeted transgene addition can provide persistent gene expression while circumventing the gene silencing and insertional mutagenesis caused by viral vector mediated random integration. This protocol describes a universal and efficient transgene targeted addition platform in human iPSCs based on utilization of validated open-source TALENs and a gene-trap-like donor to deliver transgenes into a safe harbor locus. Importantly, effective gene editing is rate-limited by the delivery efficiency of gene editing vectors. Therefore, this protocol first focuses on preparation of iPSCs for transfection to achieve high nuclear delivery efficiency. When iPSCs are dissociated into single cells using a gentle-cell dissociation reagent and transfected using an optimized program, >50% cells can be induced to take up the large gene editing vectors. Because the AAVS1 locus is located in the intron of an active gene (*PPP1R12C*), a splicing acceptor (SA)-linked puromycin resistant gene (PAC) was used to select targeted iPSCs while excluding random integration-only and untransfected cells. This strategy greatly increases the chance of obtaining targeted clones, and can be used in other active gene targeting experiments as well. Two weeks after puromycin selection at the dose adjusted for the specific iPSC line, clones are ready to be picked by manual dissection of large, isolated colonies into smaller pieces that are transferred to fresh medium in a smaller well for further expansion and genetic and functional screening. One can follow this protocol to readily obtain multiple GFP reporter iPSC lines that are useful for *in vivo* and *in vitro* imaging and cell isolation.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52504/>

Introduction

The ability to reprogram human somatic cells into embryonic stem cell-like induced pluripotent stem cells (iPSCs) was first discovered by Takahashi *et al.* in 2007¹. Human dermal fibroblasts transduced with retroviruses expressing four transcription factors (The so-dubbed Yamanaka factors Oct3/4, Sox2, c-Myc, and Klf4) were shown to be highly similar to human embryonic stem cells (hESCs) based on morphology, proliferation, gene expression, and epigenetic status; crucially, iPSCs are also capable of differentiating into cells of all three germ layers¹. The proliferative potential and differentiation capacity of iPSCs makes them very attractive tools; by reprogramming cells from patients suffering from specific diseases, iPSCs can be used both as *in vitro* disease model systems and as potential therapeutics.

For the latter purpose, several issues must be addressed before the full potential of iPSCs in a clinical setting can be realized; the tumorigenic potential of *in vitro* cultured hESCs and iPSCs, the use of xenogenic derivatives during reprogramming and cell maintenance, and the need to track transplanted cells *in vivo* are all crucial hurdles to the clinical application of pluripotent stem cells (Reviewed by Hentze *et al.*²). An ideal solution to the need for tracking differentiated cells post-transplantation would involve a visually detectable marker that resists silencing and variegation regardless of the application. Robust and sustained expression of integrated transgenes is most readily achievable when exogenous DNA is introduced into safe-harbor loci; that is, genomic sites that enable sufficient transcription of an integrated vector while at the same time mitigating perturbations of expression in neighboring genes³. One such site that has been very well characterized since its discovery is the adeno-associated virus integration site 1 (AAVS1), in the first intron of the protein phosphatase 1 regulatory subunit 12C (*PPP1R12C*) gene. This locus has been shown not only to permit sustained and robust expression of integrated transgenes through extended time in culture and *in vitro* differentiation³, but also to protect surrounding genes from transcriptional perturbation⁴; both features are thought to be due to the presence of endogenous chromatin insulator elements flanking the AAVS1 site⁵.

Advances in genome engineering tools over just the past decade have greatly facilitated the ease and efficiency with which genetic manipulations in any cell type can be achieved. While early successful experiments relied on exceedingly low levels of endogenous homologous recombination (HR) with an introduced donor to achieve gene targeting in ESCs^{6,7}, the use of site-specific nucleases, such as zinc finger nucleases (ZFNs), that significantly induce homologous recombination through the generation of a double-stranded DNA break has greatly increased the efficiency of such experiments^{8,9}. The repurposing of both transcription activator-like effectors (TALEs) of plant pathogenic

xanthomonas genera and the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system into efficient site-specific designer nucleases has made gene targeting in pluripotent stem cells an accessible and practicable methodology¹⁰⁻¹³.

A recent paper described an efficient method for the stable integration of a green fluorescent reporter cassette into the AAVS1 safe-harbor locus in human iPSCs using TALE nucleases (TALENs)¹⁴. These targeted iPSCs maintained their fluorescence even after directed differentiation to cardiomyocytes and transplantation into a mouse model of myocardial infarction (MI), providing strong evidence for the utility of such stably fluorescent pluripotent stem cells¹⁴. To obtain targeted colonies, a gene-trap method was used wherein a splicing-acceptor (SA), 2A self-cleaving peptide sequence places the puromycin N-acetyl-transferase (PAC) gene under the control of the endogenous *PPP1R12C* promoter; thus, only iPSCs that have incorporated the DNA donor at the AAVS1 locus express PAC, rendering them selectable based on puromycin-resistance; (Figure 1,¹⁵). This protocol details the procedures of generating AAVS1-GFP iPSCs reported in the recent paper¹⁴, including the process of transfecting iPSCs with TALENs and a 9.8 kb donor to integrate a 4.2kb DNA fragment into the AAVS1 safe-harbor locus, selecting iPSCs based on puromycin-resistance, and picking colonies for clonal expansion. The techniques described herein can be applied to many genome engineering experiments.

Protocol

1. Preparation of Basement Membrane Matrix and Coating of Plasticware

1. Place the frozen basement membrane matrix stock from -20 °C onto ice and thaw overnight at 4 °C.
2. After thawing, pipette 2 mg aliquots of basement membrane matrix into pre-chilled eppendorf tubes. Store these at -20 °C until needed.
3. To prepare basement membrane matrix-coated plates, thaw one aliquot on ice until the last piece of ice in the eppendorf tube disappears (usually within ~2 hr).
4. After thawing, add basement membrane matrix to 12 ml of cold (4 °C) DMEM/F12 to make basement membrane matrix coating solution.
5. Add basement membrane matrix solution to the appropriate culture vessel. For a 6-well plate, dispense 1 ml per well. Swirl the plate to make sure the basement membrane matrix solution completely covers each well.
6. Parafilm seal the basement membrane matrix-coated plate/dish, and incubate at room temperature for 1 hour before use. Alternatively, store basement membrane matrix-coated plates/dishes at 4 °C and use within 2 weeks of coating.
NOTE: Add extra DMEM/F12 to the basement membrane matrix-coated plate/dish to prevent drying. Before using 4 °C stored basement membrane matrix-coated plate/dish, place it in a biological safety cabinet and allow it to come to room temperature for at least 30 min.
7. Aspirate basement membrane matrix completely before the addition of medium and cells.

2. Preparation of E8 medium

1. Prepare E8 culture medium by thawing E8 supplement overnight at 4 °C.
2. Remove 10 ml of E8 basal medium from the 500 ml stock and discard.
3. Pipette the entire 10 ml vial of E8 supplement directly into 490 ml of E8 basal medium. Do not warm complete E8 medium in a 37 °C water bath, as repeated temperature fluctuations can degrade the bFGF in the complete E8 medium.
4. Use complete E8 medium within 2 weeks of addition of the supplement.

3. Thawing of iPSCs

1. Remove a vial of frozen iPSCs from liquid nitrogen and place on dry ice.
2. Rapidly thaw the vial in a 37 °C water bath; swirl the vial in the water bath until only a tiny fragment of ice remains.
3. Spray the vial with 70% ethanol and transfer to a biological safety cabinet.
4. Add 1 ml of room temperature E8 medium dropwise directly to the vial.
5. Using a 2 ml pipette, transfer the cell suspension dropwise into 9 ml of E8 medium in a 15 ml conical tube. Swirl the tube frequently to ensure that the cells and medium mix well quickly.
6. Centrifuge the cells at 200 x g for 5 min.
7. Aspirate the supernatant, and resuspend the cell pellet in an appropriate volume of E8 supplemented with 10 µM Y-27632.
8. Add the cells to an appropriate number of basement membrane matrix-coated wells, and place in a 37 °C, 5% CO₂ incubator overnight. It is recommended to plate at least 0.2 x 10⁶ iPSC per well of a 6-well plate to enable quick recovery after thawing.

4. Maintenance and Routine Passaging of iPSCs

1. Refresh E8 medium daily.
2. Monitor the morphology and confluency of cells with an inverted microscope. iPSCs of high quality grow in flat colonies with distinct borders; individual colonies possess a "cobblestone-like" appearance.
3. Passage the iPSCs cells when they reach ~70% confluency.
4. Prepare an EDTA passaging solution by adding 0.9 g NaCl and 500 µl 0.5 M EDTA to 500 ml DPBS. Mix well to dissolve NaCl, and vacuum filter to sterilize. Warm an aliquot of the passaging solution in a 37 °C water bath prior to passaging.
5. To passage, aspirate spent culture medium and wash cells once with an equal volume of warm passaging solution. Aspirate, and pipette enough EDTA passaging solution to coat the cells (1 ml per well of a 6-well plate).
6. Place the cells under an inverted microscope and observe the iPSC colonies. The appearance of holes within colonies and raised borders should become apparent within 2 to 5 min.
7. Carefully aspirate the EDTA passaging solution.
8. Using a 10 ml pipette, dispense 4 ml of E8 medium (if using a 6-well plate) under high pressure directly into each well to be passaged.

9. Collect the iPSC clumps, and split into an appropriate number of wells depending on the split ratio from 1:8 to 1:12. Do not over-pipette, as disaggregation of cell clumps will result in poor viability.
10. Place the plate in an incubator, and rock the plate back-and-forth and side-to-side several times to disperse the cells.

5. Preparation of MEFs and iPSCs for Transfection

1. 48 hr prior to transfection, passage iPSCs at ~1:6 into four or more wells of a basement membrane matrix-coated 6-well plate, so that they will be 70% confluent two days hence.
2. The next day, thaw DR4 MEFs into MEF medium consisting of DMEM (high glucose) supplemented with 10% FBS and 1x MEM-NEAA.
3. Plate DR4 MEFs into two 10-cm dishes at $\sim 2 \times 10^4$ cells/cm² and incubate overnight at 37 °C.
4. On the day of transfection, change MEF medium to E8 supplemented with 10 μ M Y-27632 30 min before performing transfection on iPSCs.
5. Optional: If flow cytometric analysis of iPSCs post-transfection is desired, remove a basement membrane matrix-coated plate from 4 °C and place at room temp.
6. Optional: 4 hr prior to transfection, supplement pre-transfection iPSC culture with Y-27632 at the final concentration of 10 μ M.

6. Gentle-cell Dissociation Reagent Treatment and Transfection of iPSCs using an Electroporation System

1. Remove P3 primary cell transfection solution from 4 °C and allow it to come to room temperature for ~30 min. Add the entire 100 μ l supplement to the transfection solution prior to use.
2. Warm gentle-cell dissociation reagent in a 37 °C water bath.
3. Obtain AAVS1 TALENs (pZT-AAVS1-L1 and pZT-AAVS1-R1) and AAVS1-CAG-EGFP donor from -20 °C.
4. Remove iPSCs from the incubator and wash once with DPBS.
5. Add 1 ml of gentle-cell dissociation reagent per well, and incubate iPSCs at 37 °C for 5 min, or until greater than 50% of the cells have dissociated from the culture vessel.
6. Pipette the cells up and down a few times using a p1000 pipette to dissociate any remaining cells from the culture vessel, and to break up iPSC clumps.
7. Add 2 ml of E8 medium to each well, and pipette up and down several times using a 10 ml pipette to further disaggregate cell clumps into single cells
NOTE: Transfection efficiency declines significantly if cell clumps are not sufficiently disaggregated.
8. Collect iPSCs in a 15 ml conical tube, and centrifuge at 100 x g for 3 min.
9. Aspirate supernatant, and resuspend cells in a minimal amount of E8 medium.
10. Count the cells using a hemocytometer after application of a vital stain such as 0.4% Trypan blue. Ensure that cells are sufficiently dissociated while counting (1-3 cells per "clump").
11. Dispense 3×10^6 cells into each of two 15 ml conical tubes, and spin down again at 100 x g for 3 min.
NOTE: Low speed centrifugation reduces the cell stress and allows easy re-suspension of iPSCs before electroporation.
12. Set the electroporation system to the cell-type specific program for the human embryonic stem cell line H9 (Program CB-150).
13. After centrifugation, aspirate the supernatant from the cell pellets. To one pellet, add 10 μ g of the HR donor as control sample. To the other pellet add 10 μ g of the HR donor, along with 5 μ g of each TALEN (pZT-AAVS1-L1 and pZT-AAVS1-R1) as experimental sample.
14. Resuspend each cell pellet in 100 μ l of P3 primary cell transfection solution, and transfer to a cuvette.
15. Perform the transfection and immediately add 500 μ l of room temperature E8 medium to each cuvette.
16. Transfer the transfected iPSCs dropwise to one 10-cm dish containing DR4 MEFs prepared in step 5.4.
17. Optional: Add several drops from each experiment into one well of a basement membrane matrix-coated 6-well plate if flow cytometry analysis of transfection efficiency is desired.
18. Repeat steps 6.15-6.17 to finish both samples. The following day, wash cells with DPBS twice and switch culture medium to NutriStem, which appears to support iPSC culture on feeders better than E8.
19. Transient EGFP expression peaks at 48 to 72 hr post-transfection; assess transfection efficiency as desired.

7. Puromycin Selection of Targeted iPSCs

1. Begin puromycin selection 72 hr after transfection when the iPSCs reach 70% confluency.
2. For NCRM5 iPSCs, begin puromycin selection at 0.5 μ g/ml puromycin (1/2 the full dose) diluted into NutriStem culture medium. The optimal puromycin concentration may vary from 0.25 to 1 μ g/ml for some iPSC lines.
3. Culture iPSCs in NutriStem + 0.5 μ g/ml puromycin for 3 days, refreshing the medium daily.
4. After 3 days, increase the concentration of puromycin to 1 μ g/ml.
5. Culture iPSCs under 1 μ g/ml puromycin selection for another 7 to 8 days, or until distinct colonies appear large enough for colony picking.

8. Colony Picking and Expanding of Targeted iPSCs

1. Use basement membrane matrix to coat a 96-well plate by dispensing 50 μ l of basement membrane matrix coating solution (2 mg basement membrane matrix diluted into 12 ml DMEM/F12) into each well. Store the plate at 4 °C overnight before use.
2. After allowing the basement membrane matrix-coated plate to come to room temperature, aspirate the basement membrane matrix and dispense 100 μ l E8 supplemented with 10 μ M Y-27632 into each well.
3. Place an inverted microscope into a biological safety cabinet, and spray lightly with 70% EtOH to sterilize.
4. Pull a glass Pasteur pipette over a Bunsen burner to obtain an ideal colony-picking tool that has a tiny "hook" at the tip. Sterilize with 70% EtOH, and place in the hood to dry.
5. Remove the dish containing targeted iPSC colonies from the incubator, and place into the hood under the microscope.

6. Pick iPSCs by tracing a circle around the colony border with the colony-picking tool.
7. Use the "hook" of the colony-picking tool to gently scrape and remove the iPSC colony from surface of the plate. For larger colonies, drawing an X with the colony-picking tool to quarter the colony will facilitate cell growth after re-plating.
8. Once cell clumps are detached and freely floating, use a p200 pipette set to ~30 μ l to collect the iPSCs. Plate cells directly into the 96-well plate.
9. Optional: use a p20 pipette set to ~5 μ l to collect the iPSCs into a eppendorf tube, then add 50 μ l gentle-cell dissociation reagent to digest for 5 min at 37 °C. After digestion, add 500 μ l E8 medium into the eppendorf tube to dilute the gentle-cell dissociation reagent and spin down the cells at 200 x g in a standard table-top microcentrifuge for 5 min. Then aspirate most of the medium and leave ~30 μ l to resuspend and transfer the cells into the 96-well plate.
NOTE: This approach will facilitate the dissociation of the cell clump and quick expansion of picked colony.
10. Continue colony picking until a sufficient number of iPSC colonies have been collected (roughly 20-30 colonies per experiment is recommended).
11. After colony picking, place the 96-well plate in an incubator overnight. Refresh with complete E8 medium the following day, and culture until ~70% confluent.
12. Passage cells from the 96-well into a 24-well, then into a 6-well, as described in steps 4.5-4.10.
NOTE: the volumes of EDTA solution and E8 medium should be proportionally reduced when using wells smaller than a 6-well plate.
13. Assess targeting success by junction PCR and Southern blot analysis to confirm targeted cassette integration and absence of randomly inserted vectors¹⁶.

Representative Results

A visualization of the protocol is provided in **Figure 2**, with periods during which iPSCs are cultured in different medium highlighted by either green for E8 or blue for NutriStem. It is important to transfect only high-quality iPSCs; examine culture dishes throughout routine maintenance and verify that iPSC cultures contain mainly distinct colonies bearing a cobblestone-like morphology (**Figure 3A**); differentiated cells should not occupy more than 10% of the culture. Transfectability of iPSCs is assessed and optimized using the small pMax-GFP vector (**Figure 4A-B**), as transfection with pMax-GFP typically represents the maximum achievable efficiency due to its small size. For example, we achieved 68.6% transfection efficiency with pMax-GFP (**Figure 4B**). To target the AAVS1 safe-harbor, iPSCs are passaged using a gentle single-cell dissociation reagent and are transfected with AAVS1-TALENs and AAVS1-CAG-EGFP (plasmids depicted in **Figure 1A**). Transfected iPSCs are then plated on a suitable density of DR4 MEFs (**Figure 3B**). Transient expression of AAVS1-CAG-EGFP peaks at 48 to 72 hours post-transfection (**Figure 3C**); if desired, a small portion of transfected iPSCs can be FACS analyzed. NCRM5 iPSCs can be transfected with the efficiency of 60.9% using AAVS1-CAG-EGFP donor (**Figure 4C**). Transfection efficiency can vary greatly; for experiments wherein the GFP+ fraction is even as low as 10%, continue to puromycin selection. After performing puromycin selection, individual clones displaying uniform fluorescence should be large enough for colony picking (**Figure 3D**). To pick clones, a suitable colony is located under low magnification (**Figure 3E**) and is isolated by first tracing and quartering it (**Figures 3F-G**). The quartered colonies are then gently scraped from the culture vessel (**Figure 3H**). Using a p200 pipette, cell clumps are then transferred into one well of a basement membrane matrix-coated 96-well plate. iPSCs should attach within 2-4 hours of plating (**Figure 3I**). If attachment is not observed within 24 hours, Basement membrane matrix-coating or Y-27632 treatment were likely sub-optimal; Basement membrane matrix-coat a new 96-well plate and prepare fresh E8 + 10 μ M Y-27632 before picking any more colonies. Once in a 96-well format, targeted iPSC clones are expanded and should exhibit stable and uniform expression of GFP (**Figures 4D-E**).

Figure 1: Gene targeting vectors and targeting schematic. (A) Representations of the AAVS1-CAG-EGFP, and pZT-AAVS1-L1/R1 TALENs plasmids used in this study. The SA-2A element in the AAVS1-CAG-EGFP plasmid represents the splice-acceptor and 2A self-cleaving peptide used to restrict puromycin N-acetyl-transferase (PAC) gene expression to targeted-integration events. The chicken β-actin globin (CAG) promoter is used to drive expression of EGFP. (B) The AAVS1 safe-harbor, contained within Intron 1 of the *PPP1R12C* gene, is targeted using TALENs to generate a double-stranded DNA break. This activates the homologous recombination (HR) repair machinery, which then uses the AAVS1-CAG-EGFP donor (bearing homology arms flanking the cut site) as a substrate for repair. The cassette is integrated and the PAC gene is placed under the control of the endogenous *PPP1R12C* promoter.

Figure 2. Timeline of gene targeting experiment. iPSCs are cultured to 70% confluency and passaged ~1:6 on day -2 (d-2). MEFs are plated at d-1, and iPSCs are collected and transfected at d0. At d1, media is switched to NutriStem, and iPSCs are cultured for two more days before puromycin is added to the medium at d3. Colonies are typically ready for picking by d12. Periods during which iPSCs are cultured in E8 medium are highlighted green, while periods of NutriStem culture are in blue.

Figure 3: iPSC gene targeting representative images. (A) Phase image of high quality iPSCs. Note the phase-bright border and cobblestone-like morphology. (B) DR4 MEFs plated at 2×10^4 cells/cm² twenty-four hours after thawing. (C) Seventy-two hours post-transfection, EGFP+ cells are clearly apparent when viewed under a fluorescence microscope. (D) After puromycin-based selection for ~14 days, colonies displaying uniform GFP fluorescence are large enough to be picked. (E-H) Representative images of the colony picking process. Colonies are picked using a pulled Pasteur pipette, first by outlining the colony, and then by quartering it to obtain smaller cell clumps. (I) Picked iPSC colonies attach to the basement membrane matrix-coated surface of the 96-well plate within 2 to 4 hr. [Please click here to view a larger version of this figure.](#)

Figure 4: FACS analysis of transfection efficiency and clonal targeted iPSCs. (A) Control NCRM5 iPSCs without transfection of any plasmid. (B) NCRM5 iPSCs transfected with the small test vector pMax-GFP are FACS analyzed for GFP expression. (C) Transient expression of the AAVS1-CAG-EGFP plasmid is assessed by FACS. (D) An NCRM5-AAVS1-CAG-EGFP clone displays uniformly positive EGFP expression as assayed by FACS. Note the tight clustering of analyzed iPSCs as compared to C). (E) Fluorescence image of an expanded NCRM5-AAVS1-CAG-EGFP targeted clone.

Discussion

The most critical steps for the successful generation of AAVS1 safe-harbor targeted human iPSCs are: (1) efficiently delivering TALEN and donor plasmids into iPSCs by transfection; (2) optimizing dissociation of iPSCs into single cells before transfection and plating density after transfection; (3) optimizing dose and time of drug-selection based on the growth of iPSC line; (4) carefully dissecting and picking targeted colonies and transferring to new plate/well. Compared to similar methods used in Hockemeyer's paper¹⁰, this protocol used a pair of open-source

AAVS1-TALENs, different iPSC dissociation reagent, and different transfection device to deliver TALENs and donor vectors, which helped to reduce the number of iPSCs used in the experiment while achieving high transfection and targeting efficiencies.

To achieve high-efficiency gene editing in human iPSCs, it is essential to first optimize the delivery of gene editing reagents (DNA/RNA), while balancing the acute cell death the delivery method and reagents cause to iPSCs. The goal is to maximize the delivery efficiency while maintaining a tolerably low level of cell death. Since it is very easy to prepare large quantities of AAVS1-TALEN plasmids that can achieve >50% HR efficiency with the help of drug-selection in human iPSCs, it is not necessary to make mRNAs from TALEN plasmids. The challenge of delivery comes from large donor plasmids, which in this case is ~10 kb. It is recommended to use the AAVS1-CAG-EGFP donor to test delivery efficiency in specific human iPSC lines rather than using pMaxGFP included in the transfection kit, because pMaxGFP is a ~3.5 kb small plasmid and very easy to deliver into any cells. AAVS1-CAG-EGFP donor can be modified using restriction enzymes shown in **Figure 1A** to target different transgenes into AAVS1 locus. Another 12 kb donor, which contains a 6.4 kb cassette to replace CAG-EGFP, has been used successfully to achieve similar transfection and targeting efficiency (data not shown). In general, human iPSCs are among the difficult-to-transfect cell types and the delivery efficiency for large plasmids can be as low as 5-10% as measured by flow cytometry analysis of GFP + cells. Further optimization of transfection efficiency is dependent on the proper dissociation of human iPSCs into single cells, because cell clumps will reduce the chance of delivering gene editing reagents into each cell. This protocol uses a gentle and fast-working cell dissociation reagent, but treating iPSCs for more than 10 minutes is not recommended. Usually the iPSC culture is less than 80% confluent if following step 5.1, making a 5 minute incubation with pre-warmed gentle-cell dissociation reagent typically sufficient to dissociate the iPSCs into single cells with gentle pipetting. If not all the cells can be dissociated into single cells after 10 min of treatment because cell culture is over-confluent or the colonies become very compact, simply collect the recommended number of single cells from more wells/plates and leave the tightly attached cells behind. Heavy pipetting is not recommended because mechanical shearing is more detrimental to the cells than enzymatic dissociation. When working with early-passage iPSCs (before passage #15), gentle dissociation practice is very important for cell survival because the cells are already more sensitive to transfection stress than late passage iPSCs. If it is difficult to achieve high-efficiency in both transfection and cell survival, choose the practice that gives highest transfection efficiency. After transfection, the cells should be plated at the density that will reach 50-80% confluency at day 3 when drug-selection starts. Since each iPSC line grows differently, the post-transfection plating density might need to be optimized for each cell line. A high plating density may lead to over-confluency that reduces the efficacy of drug-selection, while an extreme low plating density may cause delayed recovery and growth of drug-resistant colonies. Usually 3 million starting iPSCs are enough to be plated into 1/2 to 2 10 cm dishes depending on the survival and growth of specific iPSCs. Similarly, if using a newly generated or as-yet untested iPSC line, generating a puromycin kill curve to establish the lowest effective dose in untransfected cells may be necessary; iPSC lines can vary considerably in their sensitivity to puromycin selection. The MEFs were used for selection because they appear to support iPSC growth better than some extracellular matrices during drug selection. As a rule of thumb, a minimum of 5 days of puromycin at 1 µg/ml or 7 days at 0.5 µg/ml are needed to complete selection. Finally, colony-picking technique is crucial to preserve all the targeted colonies after drug selection. Gentle pipetting or dissociation reagent treatment helps to break the colonies into multiple pieces evenly distributed into the new well, and therefore can speed up colony expansion. If using a dissociation reagent to disaggregate the picked colonies prior to plating, make sure it is sufficiently diluted with >10x medium after treatment, because the residual dissociation reagent could kill the transferred cells if left on overnight. No matter which technique is used, practicing colony picking before the real experiments is highly encouraged.

As the described strategy utilizes a gene-trap method to drive expression of the PAC gene off of the endogenous *PPP1R2C* promoter, picking significantly more than 30 colonies should not prove necessary. The SA-2A linked PAC selection theoretically eliminates random integration-only cells, but additional random integration(s) can occur in iPSCs bearing successful targeted integrations. In most cases, nearly 100% of drug-selected clones have targeted integrations and ~10-40% of them have additional random integrations. The majority of targeted clones tend to have single-allele targeting, although experiments wherein >50% double-allele targeting does occur. The variable frequency of additional random integrations, as well as the ratio of single- versus double-allele targeting, is difficult to control. Therefore, picking ~20 colonies is recommended to ensure single- or double-allele targeting-only clones can be obtained. In light of unsuccessful targeting experiments, re-assessment of the target cells' transfectability, survival, and growth rates, the efficacy of nucleases in the specific cell line, and the quality of donor and nuclease preparations is highly recommended before re-attempting the experiment in its entirety.

It should be noted that similar gene-trap donor strategies can be used for targeting other active genes, but is not suitable for silent gene targeting which requires an independent promoter to drive selectable gene expression. Also, while the AAVS1 safe harbor is considered to have open chromatin structure, this does not guarantee that any transgene can be expressed strongly at this locus. Indeed, our recent report showed that several weak promoters were unable to drive detectable fluorescent reporter gene expression after targeted integration at AAVS1 locus¹⁴.

This protocol focuses on using well-validated TALENs to target a well-studied safe harbor locus in the human genome. The techniques are highly reproducible and easily adaptable to many applications involving any transgene. Compared to genome engineering methods using random integrations, AAVS1-TALEN mediated gene targeting is highly efficient and specific, and in the case of iPSCs, results in stably fluorescent cells that have the potential to expand and differentiate into any human cell type. While this protocol does not describe the design and validation of designer nucleases or the assessment of targeted iPSC colonies for proper cassette integration and off-target analysis, several excellent protocols have described these aspects of the methodology in detail¹⁶⁻¹⁸. The feeder-free iPSC culture and passaging techniques using E8 medium are also described in details in previous publication¹⁹. The above protocol, while optimized for gene addition into the AAVS1 safe-harbor of human iPSCs, can serve as a general template for experiments involving site-specific nuclease-mediated gene editing/addition using a homologous recombination donor in any cell type.

Disclosures

The authors declare that they have no competing financial interests.

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