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Generation of an induced pluripotent stem cell line with the constitutive EGFP reporter

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Abstract

The discovery of induced pluripotent stem cell (iPSC) technology has provided a versatile platform for basic science research and regenerative medicine. With the rise of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) systems and the ease at which they can be utilized for gene editing, creating genetically modified iPSCs has never been more advantageous for studying both organism development and potential clinical applications. However, to better understand the behavior and true therapeutic potential of iPSCs and iPSC-derived cells, a tool for labeling and monitoring these cells *in vitro* and *in vivo* is needed. Here, we describe a protocol that provides a straightforward method for introducing a stable, highly expressed fluorescent protein into iPSCs using the CRISPR/Cas9 system and a standardized donor vector. The approach involves the integration of the EGFP transgene into the transcriptionally active adeno-associated virus integration site 1 (AAVS1) locus through homology directed repair. The knockin of this transgene results in the generation of iPSC lines with constitutive expression of the EGFP protein that also persists in differentiated iPSCs. These EGFP-labeled iPSC lines are ideal for assessing iPSC differentiation *in vitro* and evaluating the distribution of iPSC-derived cells *in vivo* after transplantation into model animals.

Keywords

CRISPR/Cas9; Fluorescent protein; Induced pluripotent stem cells; Nucleofection; iPSCs

1. Introduction

The technology that allows for the generation of induced pluripotent stem cells (iPSCs) from somatic cells provides an ideal platform for disease modeling, drug discovery and therapy development. iPSCs have the potential to model the diversity of genotypes within the human population, as these cells can be generated from both normal and disease-associated somatic

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human cells (1). When combined with gene editing using programmable nucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), iPSC technology becomes even more valuable for studying gene function and for lineage tracing analyses (2, 3). The ability to correct mutations or introduce de novo mutations into otherwise healthy iPSCs further broadens iPSC applications in modeling human diseases and developing novel cell therapeutics (2). To better understand the behavior and true therapeutic potential of iPSCs, the functionality and distribution of iPSC-derived cells need to be assessed following the transplantation of these cells into model organisms. This can be achieved by the generation of iPSC lines with constitutive expression of a fluorescent protein that also persists in differentiated iPSCs. In this chapter, we present a protocol that generates iPSCs with the constitutive enhanced green fluorescent protein (EGFP) reporter using the CRISPR/Cas9 system to enable the visualization of these cells and their derivatives with the use of fluorescence microscopy.

Using CRISPR/Cas9, the generation of fluorescently labeled iPSCs has become a relatively efficient and straightforward process. The CRISPR/Cas9 system is composed of 2 main components: the Cas9 endonuclease that can introduce a DNA double stranded break (DSB) at a specific genomic location and a guide RNA (gRNA) that directs Cas9 to a predetermined genomic locus (4). The gRNA consists of a short variable 20-nucleotide sequence that is complementary to the DNA sequence being targeted and a constant region with several stem loop structures that serve as scaffolding for Cas 9 binding. The gRNA recognizes the complementary sequence on the genomic DNA upstream of the three base protospacer adjacent motif (PAM), preferably 5'-NGG-3' (where "N" can be any nucleotide base followed by two guanine nucleobases), and directs the endonuclease Cas9 to introduce a site-specific DSB. In the presence of a DNA donor template that contains a sequence of interest flanked by regions homologous to those adjacent to the Cas9 cleavage site, the DSB can trigger homology directed repair (HDR) to incorporate the exogenous DNA sequence, such as the EGFP reporter, into the predetermined genomic locus of iPSCs (5, 6). An important criterion for the successful integration of a fluorescent reporter into iPSCs is the selection of an appropriate integration site. The adeno-associated virus site 1 (AAVS1) locus has long been used a safe harbor for transgenic integration and has been shown to be transcriptionally active in multiple organs in humans, similar to the ROSA26 locus in mice (7, 8). Incorporation of the EGFP transgene into the AAVS1 locus allows for robust and stable expression in a variety of cell types and ensures continuous and stable expression in iPSCs and iPSC-derived cells following differentiation and transplantation into animal models.

There are several approaches to deliver the CRISPR/Cas9 complex into the cells of interest: (1) DNA plasmids encoding both the Cas9 protein and the gRNA; (2) mRNA encoding Cas9 alongside a separate gRNA; and (3) the Cas9 protein preassembled with the gRNA into a ribonucleoprotein (RNP) complex (4). Although the CRISPR/Cas9 complex was originally delivered via plasmid DNA, the advances in the generation of mRNA, particularly modified mRNA (mod-mRNA), which is characterized by lower cellular immunogenicity than unmodified mRNA (9, 10), and RNP complexes (4) allow for a more efficient and controllable way to introduce functional Cas9 into cells. In addition, both mod-mRNA and RNP complexes are readily available through commercial sources. The provided protocol for

the introduction of the EGFP reporter into the AAVS1 locus of human iPSCs relies on using mod-mRNA encoding the Cas9 nuclease delivered together with plasmid expressing the AAVS1-specific gRNA. A DNA plasmid with homology arms targeting the AAVS1 locus is used as a donor template for HDR to introduce the constitutive EGFP transgene. Additionally, the targeting plasmid employs a gene-trap strategy to convey puromycin resistance for positive selection of transfected cells (Fig. 1). This donor plasmid has been previously used to knockin EGFP into AAVS1 using zinc-finger nucleases (11). The AAVS1-specific gRNA, which has been previously used for modification of the AAVS1 locus together with Cas9 (12), is expressed from the human U6 polymerase III promoter of the DNA plasmid delivered together with Cas9 mod-mRNA and the donor template. Nucleofection is used to deliver the plasmids and Cas9 mod-mRNA. Nucleofection is followed by selection with puromycin to eliminate nontargeted iPSCs, since any cell that does not fully incorporate the donor construct into the AAVS-1 locus will not survive puromycin treatment. Following selection with puromycin and outgrowth, iPSC colonies with uniform EGFP expression can be manually picked and expanded for downstream applications (*see* Fig. 2a for the schematic of the protocol). Although the protocol describes the generation of iPSCs with the constitutive EGFP reporter, a similar procedure can be used for the introduction of any other marker into the AAVS1 locus of human iPSCs.

2. Materials

2.1 Targeting the AAVS1 Locus by Nucleofection

1. Matrigel human Embryonic Stem Cell (hESC)-Qualified Matrix, LDEV-free (Corning). Matrigel solidifies rapidly at room temperature (RT). Therefore, it is recommended to aliquot each new batch of the matrix upon arrival following the manufacturer's instructions. Use prechilled pipette tips, racks and tubes while working with the reagent. Store at -80°C .
2. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Thermo Fisher Scientific).
3. Tissue Culture (TC)-treated 6-well plates.
4. mTeSR1 basal medium supplemented with mTeSR1 5x supplement according to the manufacturer's instructions (mTeSR1 complete medium) (STEMCELL Technologies).
5. TrypLE Select Enzyme (1x), no phenol red (Thermo Fisher Scientific).
6. 10 mM Rock inhibitor diluted in DMSO (Y-27632 2HCl) (Selleck Chemicals).
7. Human Stem Cell Nucleofector Kit 2 (Lonza).
8. AAV-CAGGS-EGFP (11), available from Addgene (Plasmid #22212, a gift from Rudolf Jaenisch). Prepare a minimum of 500 ng/uL in Qiagen EB Buffer, store at -20°C (*see* Note 1).

¹ Bacterial stocks are grown and plasmid DNA is isolated using the Qiagen Midi Prep kit following the manufacturer's instruction.

9. gRNA_AAVS1-T2 (12), available from Addgene (Plasmid #41818, a gift from George Church). Prepare a minimum of 500 ng/uL in Qiagen EB Buffer, store at -20°C (*see* Note 1).
10. Mod-mRNA Cas9 is available commercially or can be synthesized in vitro as previously described (10). Mod-mRNA should be diluted/resuspended in Nuclease-Free Water at no less than 500 ng/ μL .

2.2. Chemical Selection for Targeted Cells

1. TC-treated 6-well plate.
2. Puromycin Dihydrochloride (10 mg/mL) (Thermo Fisher Scientific). Add 100 μL of 10 mg/mL stock solution of Puromycin to 900 μL of mTeSR1 complete medium to make a 1 mg/mL working solution (*see* Note 2).

2.3 iPSC Colony Picking and Clonal Expansion

1. mTeSR1 complete medium (STEMCELL Technologies).
2. TC-treated 6-well plates.
3. 1x DPBS (Thermo Fisher Scientific).
4. 0.5 mM EDTA in DPBS. Add 500 μL of 0.5 M EDTA to 500 mL of DPBS to create a working solution of 0.5 mM EDTA in DPBS and filter-sterilize using a 0.22 μm vacuum filtration system.

2.4 Equipment

1. Biological Safety Cabinet.
2. 37°C water bath or bead bath.
3. $37^{\circ}\text{C}/5\% \text{CO}_2/5\% \text{O}_2$ humidified tissue culture incubator (*see* Note 3).
4. Nucleofector 2b Device (Lonza).
5. Hemocytometer.
6. Centrifuge.

3. Methods

Work under RNase-free conditions and use aseptic techniques when possible. Perform all cell culture-related manipulations in a biological safety cabinet using aseptic techniques. Follow institutional biosafety standards for work with human cells. Once the nucleofection procedure is initiated, daily maintenance is required for approximately 15–17 days. Be sure to plan accordingly.

²The efficiency of puromycin may decline with repeated freezing and thawing. Prepare a 1 mg/mL working stock solution of puromycin immediately before use.

³This protocol has been optimized for iPSCs cultured in a Tri-Gas incubator set for 5% oxygen (low O_2). The procedure should work with similar efficiency for ESCs or iPSCs cultured under normoxic conditions (i.e., 20% oxygen).

3.1. Targeting the AAVS1 Locus by Nucleofection

1. Prepare 1 well of a 6-well plate of iPSCs. Confirm that iPSCs are healthy with minimal differentiation (Fig. 2b). iPSC colonies should be at approximately 75–85% confluency before the nucleofection procedure is performed. If confluency is not adequate, delay the protocol until the cells have reached the required confluency. (*see Note 4*).
2. Coat 1 well of a 6-well plate with hESC-qualified Matrigel following the manufacturer's instructions (*see Note 5*). Seal plates with parafilm and incubate for 1h at RT to polymerize inside the biological safety cabinet. Set aside.
3. Prepare an 8 mL aliquot of mTeSR1 complete medium in a 15 ml conical tube and supplement it with 10 μ M Y-27632. Invert the tube 3–4 times to mix. Place the mTeSR1 aliquot supplemented with Y-27632 in a bead bath to warm to 37°C.
4. Aspirate the spent mTeSR1 complete medium from the iPSCs to be nucleofected. Replace with 2 mL of mTeSR1 complete medium with Y-27632 prepared in step 3. Return the cells to the low O₂ incubator for 2 h. The exposure to Y-27632 may slightly change the morphology of iPSC colonies (*see Fig. 2c*).
5. After incubation, aspirate mTeSR1 complete medium with Y-27632 from the cells and add 1 mL of DPBS to rinse. Gently rock the plate to ensure complete coverage of the cells, then aspirate DPBS. Repeat the rinse with DPBS.
6. Aspirate DPBS and add 1 mL of RT TrypLE to the well. Gently rock the plate to ensure complete coverage of the cells and return the plate to the low O₂ incubator. Incubate for 3 min.
7. Remove the plate from the incubator and firmly but gently tap the side of the plate to dislodge cells. Check the cells under the microscope. If 90% of the cells are detached and floating, proceed. If the majority of cells are still attached, incubate for another 3 min. Continue to check cells every 3 minutes until 90% of the cells are detached (*see Note 6*).
8. Quickly rinse/collect the detached cells using 3 mL of mTeSR complete medium supplemented with Y-27632 from step 3 to neutralize TrypLE. Transfer the iPSC suspension into a 15 mL conical tube.
9. Invert the cell suspension 5 times to fully mix and then count the cells using a hemocytometer (*see Note 7*).

⁴iPSCs are cultured in mTeSR1 complete medium in a 6-well plate, following standard human cell culture procedures. Rock inhibitor is not used for routine passaging. One well of a 6-well plate yields approximately $1-2 \times 10^6$ cells when grown to a confluency of 75%–85%. If differentiation is present, manually pick and remove the differentiated areas, passage cells, and nucleofect when optimal confluency is reached. If the confluence of cells is higher than 85%, split cells at a lower density and proceed with the protocol when cells have reached ideal confluency.

⁵The dilution of Corning Matrigel is calculated for each lot based upon the protein concentration. Appropriately sized aliquots should be prepared according to the dilution factor provided by the manufacturer in the certificate of analysis.

⁶Do not leave TrypLE on the cells for more than 12 min, as this will greatly reduce cell viability in downstream applications. If cells are not detached within 12 min, a cell scraper can be used to mechanically dislodge iPSCs.

⁷iPSCs can remain clustered together following the treatment with TrypLE. When counting cells using a hemocytometer, estimate the number of cells in the cluster.

10. Transfer 1×10^6 iPSCs into a new 15 ml conical. Centrifuge at $200 \times g$ for 3 min at RT.
11. During centrifugation, prepare complete nucleofection solution. For each reaction, add 81.8 μ L of Nucleofector Solution to 18.2 μ L of Supplement 1 in a clean 1.5 mL Eppendorf tube (4.5:1 ratio). Pipet up and down 5 times to mix thoroughly, taking care to avoid bubbles (*see* Note 8)
12. Aspirate medium from the pelleted cells. Resuspend the pelleted cells in 100 μ L of complete nucleofection solution.
13. Add 2 μ g of Cas9 mod-mRNA, 2 μ g of AAV-CAGGS-EGFP plasmid, and 1 μ g of gRNA_AAVS1-T2 plasmid to the resuspended cells from step 12. The total volume of mod-mRNA and plasmids should not exceed 10 μ L.
14. Gently mix the complete nucleofection cell suspension by pipetting up and down, taking care to avoid bubbles. Transfer the cell suspension to the nucleofection cuvette. Pipet the solution along the side to avoid bubbles (*see* Note 9).
15. Place the nucleofection cuvette into the nucleofection chamber and execute program B-016 on the Nucleofector. Once the nucleofection is complete, add 500 μ L of mTeSR1 complete medium supplemented with Y-27632 directly into the cuvette.
16. Aspirate the Matrigel solution from the well prepared in step 2, do not allow the surface of the well to dry. Add 2 mL of prewarmed mTeSR1 complete medium supplemented with Y-27632 to the well.
17. Use the provided dropper to resuspend cells in the cuvette and gently transfer the entire transfection mix from the cuvette into the prepared well.
18. Place the plated cells into a tissue culture incubator with O₂ set to 5% (low-O₂). Once the plate is set down, disperse the cells by alternating between an up/down then left/right motion. Repeat the motions 2 more times. Do not swirl the plate to mix. Incubate the cells overnight.
19. The following day (day 2), place 2.5 mL of mTeSR1 complete medium into a bead bath to warm to 37°C. Do not supplement with Y-27632. Remove the plate with nucleofected cells from the low O₂ incubator and aspirate the spent medium. Significant cell death is expected within the 24 h following nucleofection. Add 2 mL of warm mTeSR1 complete medium and assess the expression of EGFP in cells using a fluorescence microscope. Cells should appear as small clusters with elongated and spindly morphology (Fig. 2d and 2e).
20. Replace medium with mTeSR1 complete medium 48 h post transfection (day 3), as cells continue to recover from nucleofection.

⁸. Human iPSCs are very sensitive to changes in environmental conditions. Therefore, proceed with the nucleofection steps as fast as possible

⁹. The presence of bubbles will negatively affect the nucleofection process by interfering with the ability of the solution to acquire a consistent electric charge.

3.2. Chemical Selection for Targeted Cells

1. On day 4, transfer 6.5 mL of mTeSR1 complete medium to a new 15 mL conical tube and add 1.63 μ L of a 1 mg/mL working solution of puromycin to achieve 250 ng/mL final concentration of puromycin. Close the tube and invert 5 times to thoroughly mix. Place the tube in a bead bath to warm to 37°C.
2. Remove the plate with nucleofected cells from the incubator and aspirate the spent medium. Add 2 mL of mTeSR supplemented with puromycin from step 1 and return the plate to the incubator. Closely monitor iPSCs for GFP expression and colony formation.
3. Replace mTeSR1 complete medium supplemented with puromycin daily for the next two days (days 5 and 6), for a total of 72 h of selection. Since correct targeting is a rare event, approximately 1–20 colonies should survive selection.
4. On day 7, replace the spent selection medium to fresh mTeSR1 without puromycin. Continue changing medium every day. Check cells for EGFP expression and colony formation daily. Some colonies may show mosaic expression of EGFP (Fig. 2f and 2g) while many should uniformly express the marker (Fig. 2h and 2i). Colonies will be large enough for picking and passaging approximately two weeks following nucleofection.

3.3. iPSC Colony Picking and Clonal Expansion

1. Use a fluorescent microscope to mark iPSC colonies that uniformly express EGFP for subsequent picking and expansion. The colonies should be large and clearly formed. Plan to pick as many colonies as possible - up to 12.
2. Prewarm 30 mL of mTeSR1 complete medium to 37°C.
3. Coat up to 12 wells of a 6-well plate with hESC-qualified Matrigel, depending on the number of observed colonies (*see* Subheading 3.1, step 2 for the coating procedure). Seal plates with parafilm and incubate for 1h at RT.
4. Aspirate the Matrigel solution from the coated plates and add 2 mL of mTeSR1 complete medium per well. Do not supplement with Y-27632. Do not allow the surface of the wells to dry. Set the plates aside.
5. Aspirate spent mTeSR1 complete medium from wells with targeted cells. Rinse once with 1 mL of 0.5 mM EDTA and aspirate (*see* Note 10).
6. Add 1 mL of 0.5 mM EDTA per well. Incubate for 4 min at 37°C.
7. Gently remove the plate from the incubator and place it in the biosafety cabinet (*see* Note 11).
8. Carefully aspirate EDTA from the well. Very gently add 3 mL of prewarmed mTeSR1 complete medium, taking care not to dislodge iPSC colonies.

¹⁰.To avoid cytotoxicity, EDTA must be diluted to a working stock of 0.5 mM in DPBS before using it to detach cells (*see* Subheading 2.3, item 4).

¹¹.At this point, cells may be very loosely adhered and easily dislodged.

9. Move the plate to an inverted or dissecting microscope to better visualize colonies. Prepare a sterile 1 mL pipette. Fully depress the plunger. Then, use the pipette tip and gently scrape a colony while slowly drawing liquid into the tip to collect the colony. Draw as little medium as possible while picking the iPSC colony.
10. To transfer the colony, pipet up and down 3–4 times in a single previously prepared well from step 4. Repeat steps 9–10 until as many as 12 colonies have been picked and transferred into individual wells (*see* Note 12).
11. Move plated cells back into the low O₂ incubator. To ensure even cell distribution, shake each plate back and forth and side to side. Do not swirl. Replace mTeSR1 complete medium daily.

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Abbreviations:

iPSC	Induced Pluripotent Stem Cell
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9
DSB	double stranded break
gRNA	guide RNA
HDR	homology directed repair
mod-mRNA	modified mRNA
EGFP	Enhanced Green Fluorescent Protein
PAM	protospacer adjacent motif
AAVS1	Adeno-associated virus integration site 1

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¹²Do not combine colonies into the same well; each picked colony should receive its own well.

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Fig 1. The targeting donor construct for insertion of the EGFP reporter into the AAVS1 locus of human iPSCs.

The donor DNA plasmid used to target the AAVS1 locus contains 5' and 3' homologous sequences of approximately 800 bp flanking the Cas9-induced cleavage site together with sequences encoding EGFP under the control of CAGGS promoter and puromycin resistance (11). SA, splice acceptor.

Fig. 2. The protocol for the generation of iPSCs with constitutively expressed EGFP.

a. Schematic representation of the procedures described in the protocol. **b.** Healthy iPSCs before nucleofection. **c.** A colony of iPSCs following incubation with Y-27632 on day 1 of the protocol. **d, e.** Clusters of iPSCs 24 h post-nucleofection, expressing EGFP. **f, g.** A colony of iPSCs with mosaic expression of EGFP on day 13 of the protocol. **h, i.** A colony of iPSCs uniformly expressing EGFP on day 13 of the protocol. Scale bar, 200 μm .