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Generation of GFP reporter human induced pluripotent stem cells using AAVS1 safe harbor Transcription Activator-Like Effector Nuclease

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Abstract

Generation of a fluorescent GFP reporter line in human induced pluripotent stem cells (hiPSCs) provides enormous potentials in both basic stem cell research and regenerative medicine. A protocol of efficiently generating such an engineered reporter line by gene targeting is highly desired. Transcription Activator-like Effector Nucleases (TALENs) are a new class of artificial restriction enzymes that have been shown to significantly promote homologous recombination by > 1000-fold. AAVS1 (adeno-associated virus integration site 1) locus is a “safe harbor” and has an open chromatin structure that allows insertion and stable expression of transgene. Here, we describe a step-by-step protocol from determination of TALENs activity, hiPSC culture, delivery of a donor into AAVS1 targeting site to validation of targeted integration by PCR and Southern blot analysis using hiPSC line and a pair of open-source AAVS1 TALENs.

Keywords

gene targeting; transcription activator-like effector nucleases (TALENs); adeno-associated virus integration site 1 (AAVS1); induced pluripotent stem cells (iPSCs); reporter cell line

Introduction

The discovery of human induced pluripotent stem cells (hiPSCs) significantly promotes researches in stem cell-based regenerative medicine (Rao and Malik, 2012; Walia et al., 2012). These include field of modeling human disease, screening of drug efficacy and safety, and ultimately using hiPSCs to serve as a source of autologous or allogeneic cells for cell replacement therapy. Given that hiPSCs possess such great therapeutic potential, considerable efforts have been made to develop protocols for the efficient differentiation of hiPSC into individual lineages since grafting of undifferentiated cells could result in the formation of teratomas (Deacon et al., 1998; Hentze et al., 2007). In the context of transplantation studies, a stably integrated fluorescent gene would be essential for

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monitoring cell survival, migration, and incorporation into the recipient organs. Developing such fluorescent reporter hiPSC lines that can undergo differentiation without silencing of transgenes would be of great utility.

In this protocol, we describe step-by-step procedures from hiPSC culture, measuring TALENs' gene editing efficiency through non-homologous end-joining (NHEJ) assay, delivery of an enhanced GFP(EGFP)-expressing donor into AAVS1 site by nucleofection, drug-selection of colonies, picking and expanding candidate hiPSC clones, screening targeted clones by PCR, to validation of targeted clones without random integration by Southern blot analysis using hiPSCs and a pair of open-source AAVS1 TALENs. The outline of this protocol is described in figure 1. We have used this protocol to successfully create at least ten reporter lines including EGFP lines at AAVS1 locus. The reporter hiPSCs we created using this protocol express robust and persistent EGFP in long-term cell culture, and continue to express EGFP in lineage-differentiated cells and in vivo engrafts of mice heart after cardiomyocyte transplantation.

Basic Protocol 1

T7 Endonuclease I NHEJ Assay—NHEJ is one of the major DNA repair mechanisms that happen after DNA double strand break created by TALENs, consequently resulting in error-prone mutations that are mostly small insertions and deletions (indels). NHEJ assay is designed to monitor the heterogeneous mixture of such mutations, thus measuring the enzymatic activities of TALENs to digest genomic double strand DNA. An outline of this assay is shown on fig. 2. In this protocol, we first describe nucleofection of TALENs into human 293T cells, then PCR amplification of the targeted locus followed by reannealing the amplicons and digesting mismatched heteroduplex with T7 endonuclease I (T7E1), finally resolving the digested products and analyzing the cutting efficiency by TALENs.

Materials

DMEM (Invitrogen, 11885-084)

FBS (HyClone, SH30070.03)

SF Cell Line 4D-Nucleofector™ X Kit (Lonza, V4XC-2012)

0.25% trypsin-EDTA (Invitrogen, 25200-056)

DNeasy® Blood and Tissue Kit (Qiagen, 69506)

Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, F549s)

AAVS1-TALENs (System Biosciences, GE601A-1; availability from Addgene pending)

PCR primers (AAVS1CEL-F: 5'-TTCGGGTCACCTCTCACTCC; AAVS1CEL-R: 5'-GGCTCCATCGTAAGCAAACC, Custom oligos, IDT)

QIAquick® PCR Purification Kit (Qiagen, 28104)

T7 Endonuclease I (New England BioLabs, M0302L)

4D-Nucleofector™ System (4D-Nucleofector™ Core Unit and 4D-Nucleofector™ X Unit)

PCR thermocycler

Protocol steps

Prepare 293T cells

1. Culture 293T cells with DMEM/10% FBS until 70-80% confluency in 6-well plate.

Nucleofect 293T cells with AAVS1-TALENs

- 2 Add 0.5 mL of 0.25% trypsin-EDTA for 5 min.
- 3 Add 1 mL of DMEM/10% FBS to neutralize trypsinization reaction.
- 4 Count the cells.
- 5 Transfer $\sim 1 \times 10^6$ cells into each of two 15 mL tubes. Label one as “control”; another as “cutting”.
- 6 Centrifuge the cells with 200g for 5 min.
- 7 Remove the supernatants, and the cell pellets are ready for transfection.
- 8 Start 4D-Nucleofector™ System.
- 9 Choose program CM-130.
- 10 Add the entire supplement into the 4D-Nucleofector™ solution bottle and mix thoroughly (both the supplement and solution provided by SF Cell Line 4D-hNucleofector™ X Kit).
- 11 Resuspend the cell pellet in each tube by adding 100 μ L of above 4D-Nucleofector™ mixed solution, then add 5 μ g of each AAVS1-TALEN (L1 and R1) plasmid in “cutting” tube or 10 μ L of water in “control” tube respectively.

Note that total volume of transfected substrate should not be exceeded 10% transfection mixture.
- 12 Take two Nucleofector™ cuvettes and label them as “control” and “cutting” respectively.
- 13 Transfer the above mixture in each tube into corresponding Nucleofector™ cuvette.
- 14 Gently tap the Nucleofector™ cuvettes to allow the mixture completely cover the bottom of the cuvette and remove air bubbles.
- 15 Place the cuvette into the retainer tray of Nucleofector™ X unit.
- 16 Press start to transfect.
- 17 Add 0.5 mL of DMEM/10% FBS into each cuvette immediately after nucleofection to mix the nucleofected cells well using plastic pipette provided by 4D-Nucleofector™ kit.

- 18 Transfer the transfected cells into two 6-wells of 2 mL of DMEM/10% FBS medium drop-wisely and split evenly ($\sim 0.2 - 0.5 \times 10^6$ cells per well), then label them correspondently.
- 19 Incubate the cells at 37 °C, 5% CO₂ incubator for 3 days.
- 20 Pellet both control and TALEN-treated cells at 200g for 5 min.
- 21 Isolate genomic DNA (gDNA) using DNeasy® Blood and Tissue.
- 22 Use the primers AAVS1CEL-F: 5'-TTCGGGTCACCTCTCACTCC and AAVS1CEL-R: 5'-GGCTCCATCGTAAGCAAACC to amplify a 469bp fragment around AAVS1-cutting site.
- 23 Set up a 50 µL PCR reaction using the Phusion Hot Start II High-Fidelity DNA Polymerase: 10 µL of 5× Phusion HF buffer, 1 µL (200 ng) of template DNA, 1 µL of 10 mM dNTPs, 1 µL of each primer at 10 µM, 0.5 µL of Phusion Hot Start II High-Fidelity DNA Polymerase and sterile water to 50 µL.
- 24 Run the PCR as the following: 98 °C for 2 minutes; 35× (98 °C for 10 s, 68 °C for 15 s, 72 °C 30 s); 72 °C 5 min; and hold to 4 °C.
- 25 Verify the amplification by running 8 µL of PCR reaction on a 1% agarose gel.
- 26 Purify PCR products using QIAquick® PCR Purification Kit.
Elute DNA with 25 µL of 0.1x EB.
- 27 Mix 2 µL of NEB buffer 2, 200ng purified PCR product, and water to a total volume of 19 µL.
- 28 Hybridize in a thermocycler as the following: 95 °C for 5 min, 95–85 °C at –2 °C/s, 85–25 °C at –0.1 °C/s; hold at 4 °C.

This step melts and randomly reanneals the amplicons, which convert any mutations into mismatched duplex DNA.
- 29 Add 1 µL of T7E1 (10 U) per sample.
- 30 Incubate at 37 °C for 15 min.
- 31 Add 2 µL of 2.5M EDTA to stop the reaction.
- 32 Load the samples on 1-1.5% agarose gel.

Consider using Agilent High Sensitivity DNA Kit to separate cleaved heteroduplex from parental heteroduplex if Agilent 2100 Bioanalyzer is available.
- 33 Run the gel at 100 V until the dye front reaches the end of the gel.
- 34 Capture the gel image with a UV imaging station.
- 35 Save the gel image in a format that can be quantified using image software. Here we use ChemiDoc™ XRS⁺ with image Lab™ software and save the gel image in scn format.

- 36 Reverse the image from white to black.
- 37 Choose the lane need to be quantified in the lanes menu.
- 38 Measure optical density (volume) of uncleaved band and cleaved bands.
- 39 Calculate fraction cleaved using formula: (sum of volumes of two cleaved bands)/(sum of volumes of cleaved and uncleaved PCR bands).
- 40 Estimate cutting efficiency using equation: % cutting efficiency = $100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$. See fig.3 as example.

Basic Protocol 2

Targeting Aavs1 Locus of Hipsocs With Aavs1-Talens and an Egfp Reporter

Donor—The purpose of this protocol is to generate an EGFP reporter hiPSC line in AAVS1 safe harbor locus without random integration. We first describe preparation of hiPSCs and MEFs, nucleofection of hiPSCs with specific AAVS1-TALENs and an EGFP reporter donor, then positive drug selection of colonies, picking and expanding candidate colonies for PCR screening and Southern blot analysis.

Materials

FBS defined (HyClone, SH30070.03)

DMEM with high glucose (Life Technologies, 11885-084)

MEM NEAA (100 x) (Life Technologies, 11140-076)

GlutaMax (100 x) (Life Technologies, 35060-061)

Anti-anti (100x) (Life Technologies, 15240062)

DR4 MEF 2M IRR (GlobalStem, GSC-6204G)

0.1% Gelatine (Millipore, SF008)

Matrigel (BD Bioscience, 354230)

DMEM/F12 (Life Technologies, 11330-032)

StemPro® Accutase® (Life Technologies, A11105-01)

0.5 M EDTA pH 8.0 (Mediatech, Inc., 46-034-ci)

PBS with calcium/magnesium free (Life Technologies, 14190)

Essential 8™ medium (prototype) (Life Technologies, A14666SA)

Y-27632 dihydrochloride (ROCK inhibitor, Tocris, 1254)

CryStor™CS10 (Stemcell, 7930)

DPBS (Life Technologies, 14190-144)

P3 Primary Cell 4D-Nucleofector® X kit (Lonza, PBP3-02250)

NutriStem™ XF/FF Culture Medium (Stemgnet, 01-0005)

4D-Nucleofector™ System (4D-Nucleofector™ Core Unit and 4D-Nucleofector™ X Unit, Lonza)

AAV-CAGGS-EGFP donor plasmid (Addgene, 22212)

Protocol steps

Prepare hiPSCs (day -1)

1. Culture at least 5×10^6 hiPSCs in Matrigel coated 6-well plates with 50 - 60% confluency.

Avoid 100% confluency since survival of the cells under such condition is shown lower after nucleofection.

Prepare MEF cultures (day -1)

- 2 Coat 9 wells of 6-well plate with 0.1% gelatin (1 mL/well) 4 h at 37 °C incubator.
- 3 Aspirate gelatin.
- 4 Wash the wells with DPBS (2 mL/well) 2 times.
- 5 Prepare MEF medium by mixing the following: DMEM 500 mL, MEM NEAA (100x) 5.5 mL, FBS 55 mL, GlutaMax (100x) 5.5 mL, Anti-anti (100x) 5.5 mL.
- 6 Thaw DR4 MEF 2M IRR cells in 37 °C water bath.
- 7 Transfer thawed MEF cells into 6 mL of MEF medium drop-wisely.
- 8 Centrifuge at 200g for 5 min.
- 9 Suspend the cells in 9 ml of MEF medium.
- 10 Plate the MEF cells in above gelatin-coated plates and add additional 1 mL of the MEF medium in each well. The cell number in each well is about 0.2×10^6 cells.
- 11 Grow the MEF cells in 37 °C incubator with 5 % CO₂.

Nucleofect hiPSCs with AAVS1-TALENs and EGFP reporter donor (day 0 and 1)

- 12 Wash the MEF cells with 3 mL of PBS twice.
- 13 Add 3 mL of 37 °C pre-warmed E8 medium to allow MEF cells adaptation for 4 h at 37 °C incubator.
- 14 Wash the hiPSCs in 6-well plate with 2 mL/well pre-warmed (37 °C) PBS twice.
- 15 Digest the cells in 6-well plate with 0.5 mL/well pre-warmed (37 °C) Accutase in 37 °C incubator for 3 min.
- 16 Pat the plate gently to allow the cells dislodge.

- 17 Add 5 times of volume of PBS into the digested cells and gently pipette a couple of times.
- 18 Take 20 μ L of cells to count.
- 19 Centrifuge the cells with 100 $\times$ g for 3 min.
- 20 Aspirate supernatant.
- 21 Suspend 5×10^6 hiPSCs in 100 μ L of P3 Nucleofection solution with 10 μ g of AAV-CAGGS-EGFP donor and 5 μ g of each AAVS1-TALEN plasmids.
Total volume of plasmids should not exceed 10% of transfection solution.
- 22 Transfer the mixture into nucleofection X-unit cuvette.
- 23 Nucleofect the cells in 4D-Nucleofector™ System using program CB150.
- 24 Add 0.5 mL of E8 medium into the cuvette immediately after nucleofection and mix the medium with the nucleofected cells well using plastic pipette provided by 4D-Nucleofector™ kit.
- 25 Transfer the transfected cells into MEF plates by splitting evenly into three 6-wells drop-wisely, then add 3 μ L of 10mM Y27632 in each well.
- 26 Grow the cells in 37 °C incubator for 24 h.
- 27 Check survival of transfected cells and expression of EGFP (at least 30% confluency should be observed and >30% transfected cells should be EGFP+).
- 28 Wash the cells with 4 ml/well PBS to remove dead cells.
- 29 Change to 3-4 mL of fresh NutriStem medium.
Changing from high bFGF E8 medium to low bFGF NutriStem medium will slow down the growth to prevent overgrown-induced differentiation, plus better survival during following drug selection.

Select for drug-resistant hiPSCs (day 2 to 12)

- 30 Start selection by changing to 3 mL of NutriStem medium containing 0.3 μ g/mL puromycin every day for 2 days.
- 31 Continue selection by changing to 3 mL of NutriStem medium containing 0.5 μ g/mL puromycin every day for another 8 days or until colonies grow big enough but not touching each other.

Pick colonies (day 13 to 20)

- 32 Coat one 96-well plate with 100 μ L of Matrigel per well overnight at 4 °C one day before picking colonies.
- 33 Add 10 μ L of E8 medium into 1.5 mL of sterile eppendorf tube.
- 34 Label individual and uniformly EGFP-expressing colonies with object marker mounted on objective lens turret of fluorescent microscope.

- 35 Dissect each labeled colony under dissection microscope using 10 μ L pipette tip and transfer the dissected colony into above eppendorf tube.
- 36 Add 100 μ L of Accutase and digest the colony for 5 min into single cells at 37 $^{\circ}$ C.
- 37 Add 1 mL of E8 medium into the tube.
- 38 Spin down the digested cells with 300g for 5 min.
- 39 Aspirate the medium and resuspend the cells with 200 μ L of E8 medium containing 10 μ M Y27632.
- 40 Transfer the cells into Matrigel-coated 96-well plates.
Please do 5 -10 digestions each time dependent on speed of colony-picking. The faster the better survival.
- 41 Change E8 medium every other day until $\sim$ 80% confluency.

Expand clones for PCR screening (day 21-30)

- 42 Coat two 24-well plates with Matrigel overnight at 4 $^{\circ}$ C one day before passaging colonies out of 96-well plates. Label one plate "Colonies" and another plate "PCR screening".
- 43 Warm up the both 24-well plates at culture hood for at least 30 min before passaging the colonies.
- 44 Aspirate the Matrigel solution from both 24-well plates.
- 45 Add 1 mL of E8 medium containing 10 μ M Y27632 into each well of 24-well plate.
- 46 Aspirate the medium in those 96-wells where the cells have reached $\sim$ 80% confluency.
- 47 Wash the cells with 200 μ L of DPBS.
- 48 Dissociate the clones with 100 μ L of 0.5 mM EDTA dissociation buffer for 5 – 10 min at 37 $^{\circ}$ C incubator.
- 49 Transfer half of the dissociated cells of each well from 96-well plate into the above 24-well plate labeled with "PCR screening"; remaining half into the 24-well of "Colonies" plate.
- 50 Grown the cells at 37 $^{\circ}$ C by changing E8 medium every other day until 70 -80% confluency.

PCR screening (25-30)

- 51 Harvest colonies grown in "PCR screening" plate once they reach 70-80% confluency.
- 52 Perform PCR screening using Support Protocol 1.

- 53 Identify positive integrated clones based on PCR screening results.
- 54 Label positive integrated clone in “Colonies” plate.

Southern Confirmation (day 31-35)

- 55 Passage each positive integrated clone in “Colonies” plate into 3 wells of Matrigel-coated 6-well plates with 0.5 mM EDTA passaging method.
- 56 Change E8 medium every day until ~80% confluency.
- 57 Harvest 2 wells of each positive clone and perform Southern blot analysis to confirm correct integration (see Support Protocol 1).

Cryopreserve positive integrated colons

- 58 Cryopreserve remaining 1 well of cells using CryStor™CS10 freezing medium in liquid nitrogen and label the cryovial.

Support Protocol 1

Pcr Screening and Southern Blot Confirmation of Aavs1-Targeted Integration—

Amplification of either 5' or 3'-junction of targeted transgene at AAVS1 locus by PCR provides a rapid and efficient screening method for selecting positive clones from picked drug-resistant colonies. Southern blot analysis using internal probe derived from donor vector, combining with restriction enzyme digestion pattern, further identifies AAVS1-specific integrated gene without random integration from the PCR-screened positive clones. Here, we first describe PCR screening method, followed by Southern blot analysis using DIG-dUTP-labelled probes.

Materials

- Platinum Taq (Invitrogen, 10966-034)
- PCR DIG probe synthesis kit (Roche, 11636090910)
- 5'-junction PCR primers (5'-F: 5'-CTGCCGTCTCTCTCCTGAGT; 5'-R: 5'-GTGGGCTTGTA CTGGTCAT, Custom oligos, IDT)
- Internal AAVS1 probe primers (AAVS1pb-F: 5'-GGCCTGGGTACCTCTACG; AAVS1pb-R: 5'-GAACCAGAGCCACATTAACCG, Custom oligos, IDT)
- DNA Molecular Weight Marker II, Digoxigenin-labeled (Roche, 11218590910)
- DIG Easy Hyb Granules (Roche, 11796895001)
- Anti-Digoxigenin-AP, Fab fragments (Roche, 11093274910)
- DIG Wash and Block Buffer Set (Roche, 11585762001)
- CSPD ready-to-use Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Roche, 11755633001)
- Nylon Membrane, Positively Charged, (Roche, 11209272001)
- 20x SSC (KD Medical, RGF-3240)

Denaturing Solution (KD Medical, RGF-3030)
Neutralizing Solution (KD Medical, RGF-3180)
Veriti® thermal cycler system (Life Technologies)
HL-2000 HybriLinker Hybridization Oven (UVP, LLC)
Stuart SSL 3Gyro Rock (Bibby Scientific Limited)

Protocol steps

Screen AAVS1-TALEN mediated targeted integration of transgene using junction PCR

1. Design a pair of primers for amplifying either 5'- or 3'- junction between AAVS1 genome and inserted donor. The 5'-junction PCR primers produce a 1033bp product (see fig. 4 as example).
2. Extract genomic DNA (gDNA) from all clones collected from "PCR Screening" plate using DNeasy® Blood and Tissue Kit.
3. Set up a 20 μ L PCR reaction for each sample using Platinum Taq: 2 μ L of 10 $\times$ Platinum Taq buffer, 1 μ L (200 ng) of template DNA, 0.4 μ L of 10 mM dNTPs, 0.6 μ L of 50 mM MgCl₂, 1 μ L of each primer at 10 μ M, 0.1 μ L Platinum Taq and water to 20 μ L.
4. Run the PCR as follows: 95 °C for 2 minutes; 35 $\times$ (95 °C for 40 s, 62 °C for 40 s, 72 °C 2 m); 72 °C 7 min; and hold to 4 °C.
5. Separate PCR products by running 1% agarose gel in 1x TAE buffer (see fig. 2).
6. Choose clones that show correct PCR products in both 5' and 3' junctions to further validate of integration at AAVS1 locus by Southern blot analysis.

Southern Blot analysis

Preparation of DIG-dUTP-labelled AAVS1 internal probe

- 7 Design a pair of primers for amplifying internal AAV-CAGGS-EGFP donor as a probe. The internal AAVS1 probe recognizing left homology arm is a 705bp product. (see fig. 4)

We prefer the probe that recognizes homology arms so that it can be used to simultaneously detect wild-type, targeted integration and random integration alleles. Please consider that the detected bands between wild type and targeted should be easily separated

- 8 Set up and run a 50 μ L PCR reaction using 1ng of AAV-CAGGS-EGFP donor as the template DNA and recommended PCR condition from PCR DIG Probe Synthesis Kit and include both unlabeled control probe and DIG-dUTP labeled probe.
- 9 Confirm PCR products by taking 2 μ L of PCR production to run 1% agarose gel in 1x TAE buffer.

Note: DIG-dUTP-labeled probes should migrate slower than unlabeled control probe.

- 10 Store DIG-dUTP-labeled probe in -20 °C for future use.

DNA transfer and fixation

- 11 Extract genomic gDNA from samples collected in step 47 of Basic Protocol 2 using DNeasy® Blood and Tissue Kit. (Note: Elute gDNA with $0.1 \times$ AE buffer. The concentration of gDNA should be $\sim 1 \mu\text{g}/\mu\text{L}$, the higher the better).
- 12 Digest $\sim 10 \mu\text{g}$ gDNA with proper restricted enzymes overnight at 37 °C.

Note: The total digestion volume should be equal to or less than 25 μL to fit in the lanes of small agarose gel.
- 13 Inactivate the enzyme activity by heating 10 min at 80 °C.
- 14 Add 6 μL of $5 \times$ loading buffer to each sample.
- 15 Separate digested gDNA in 0.7% small agarose gel without any DNA staining dye in a $1 \times$ TAE buffer. Use the migration of loading dye to estimate the time for sufficient separation of interested bands, such as wild-type and targeted integration.

Note: Keep one lane either first or last for loading 2 μL of DIG-labeled DNA marker II.
- 16 Denature the gDNA by incubation of the gel in the tray with 30 mL of denature buffer for 15 min twice.
- 17 Neutralize the gDNA by incubation of the gel in the tray with 30 mL of neutralization buffer for 15 min twice.
- 18 Rinse the gel in the tray with 40 mL of ddH₂O once.
- 19 Assembly the “sandwich” for capillary transfer. First place Whatman 3MM-paper on a upside down gel tray in a large container containing $20 \times$ SSC and let the sides of the paper soaked in the $20 \times$ SSC to make a paper “bridge”. On the top of the “bridge” place the gel upside down and top it by a Nylon membrane cut as the same size of the gel. Then cover the Nylon membrane by 3 layers of Whatman 3MM-paper and additional layers of paper towel. Add heavy weight on the top of “sandwich” to assist transfer.

Note: Make sure gel, nylon membrane and Whatman 3MM-paper are all wet without bubble. Cut a corner of Nylon membrane to identify the side and orientation of the membrane.
- 20 Allow gDNA transfer overnight.
- 21 Place the membrane on Whatman 3MM-paper soaked with $10 \times$ SSC.
- 22 Crosslink the gDNA to the membrane with UV-cross linker in automatic setting.

- 23 Rinse the membrane briefly in ddH₂O and allow to air-dry. Transfer the membrane to a hybridization tube/bag.

Hybridization

- 24 Pre-warm DIG Easy Hyb solution at 42 °C.
- 25 Pre-hybridize the membrane with 5 mL of pre-warmed 42 °C DIG Easy Hyb solution for 30 min in hybridization oven at 42 °C in a rolling speed of 30 rpm.
- 26 Thaw the DIG-dUTP labeled probes.
- 27 Denature the probes by heating 10 min at 95 °C.
- 28 Add the denatured probe into 3-7 mL of pre-warmed 42 °C DIG Easy Hyb solution.
Use 2-4 µL of denatured probe per mL of DIG Easy Hyb solution.
- 29 Change the pre-hybridization solution with the probe solution.
- 30 Hybridize the membrane overnight at 42 °C in a rolling speed of 30 rpm.
- 31 Pull off the probe solution and store at -20 °C for reuse.
To reuse the probe that is already diluted in the DIG Easy Hyb solution, freshly denature the mixture at 68°C for 10 min.
- 32 Wash the membranes twice with 20 mL of 2 × SSC/0.1% SDS solution for 15 min at 15-25 °C hybridization in a rolling speed of 40 rpm.
- 33 Wash the membranes twice with 20 mL of pre-warmed 65 °C 0.1 × SSC/0.1% SDS solution for 15 min at 65 °C hybridization in a rolling speed of 40 rpm.

Immunological detection

- 34 Prepare a 1 × blocking solution by diluting the 10 × Blocking solution with 1x maleic acid.
- 35 Block the membrane with 20 mL of blocking solution at 25 °C for 30 min in a rolling speed of 40 rpm.
- 36 Incubate the membrane with 10 mL of Anti-Digoxigenin-AP, Fab fragments (1:10,000) in blocking solution at 25 °C for 30 min in a rolling speed of 40 rpm.
- 37 Wash 2 × 15 min in 40 mL of Washing buffer in a tray at room temperature.
- 38 Equilibrate 3 min in 10 mL of Detection buffer in a tray at room temperature.
- 39 Place the membrane with DNA side facing up on a plastic development folder and apply 1ml CSPD ready-to-use. Immediately cover the membrane with the top sheet of the folder to spread the substrate evenly without air bubbles.
Incubate for 5 min at room temperature before squeezing out excess liquid. Seal the folder and incubate at 37 °C for 10 min to enhance the luminescent reaction. Expose to a luminescent imager for 5 – 30 min or to X-ray film for 15 – 30 min at room temperature.

Note: Luminescent signal remains constant for 24 h.

- 40 Identify integrated colonies by looking at targeted bands shown in fig. 4.
- 41 Save the membrane in 4 °C in case reuse.

Note: Do not allow it dry.

Stripping and reprobing of Southern blots

- 42 Rinse the used membrane with 50 mL of double-distilled water.
- 43 Wash the membrane with 30 mL of 0.2 N NaOH containing 0.1% SDS for 2 × 15 min at 37 °C hybridization over in a rolling speed of 40 rpm.
- 44 Rinse the membrane with 40 mL of 2x SSC at room temperature.
- 45 Prehybridize and hybridize with a second probe by following above procedures.

Reagents and Solutions

MEF medium

DMEM 500 mL

MEM NEAA (100x) 5.5 mL

FBS 55 mL

GlutaMax (100x) 5.5 mL

Penicillin/streptomycin (100x) 5 mL

Anti-anti (100x) 5.5 mL

Filter sterilize using a 0.22 µm Stericup filtration unit (Millipore)

Store up to 1 week at 4 °C.

10 mM stock Y-27632 dihydrochloride

Dissolve in H₂O to 3.3 mg/ml

Filter sterilize, aliquot and store at -20 °C

0.5 mM EDTA dissociation buffer

0.5 M EDTA 500 µL

NaCl 0.9 g

PBS calcium/magnesium free 500 ml

Filter sterilize

Store at room temperature

Commentary

Background Information

Targeted genome editing is an efficient approach to create fluorescent hiPSC lines. This technology relies on the use of engineered nuclease: a fusion protein containing a customizable sequence-specific DNA binding domain covalently linked to a non-specific DNA endonuclease domain such as one from FokI. This targetable nuclease creates a double strand break (DSB) at a specific DNA sequence, which must then be repaired by either the non-homologous end-joining (NHEJ) or the homology-directed repair (HDR) pathways. Through HDR, an exogenous fluorescent reporter DNA vector (also called donor), here that is enhanced green fluorescent protein (EGFP), is permanently integrated and expressed under the control of either an endogenous or exogenous promoter, depending on the transgene design and intended function. Some methods, such as lentiviral integration of the transgene, often result in random and more than one integration in the transduced cells (Hamaguchi et al., 2000). In contrast, sequence-specific nuclease mediated genome editing method creates a site-specific integration, enabling precision editing and control over expression levels in the targeted cells. Zinc finger nuclease (ZFN) is one example of such designer nuclease, and is composed of a nonspecific FokI domain fused to a zinc finger protein (ZFP) that usually has an array of three to four zinc finger motifs. Each motif recognizes a specific nucleotide triplet. FokI functions as a dimer. Thus, two zinc finger arrays must be designed for each target site to be able to generate DNA DSB. ZFNs have been successfully used to modify endogenous genes in a wide range of cell types including hiPSCs (Zou et al., 2009). Recently, Transcription Activator-like Effector Nucleases (TALENs) have rapidly emerged as an alternative tool to ZFNs for gene targeting. Similar to ZFNs, TALENs comprise a nonspecific FokI nuclease domain fused to a customized TALE DNA binding domain. This DNA binding domain consists of highly conserved 33-35 amino acid repeats derived from the transcription activator-like effector (TALE), a proteins secreted by *Xanthomonas spp.* proto-bacteria to alter transcription in host plant cells (Boch and Bonas, 2010). Each individual TALE repeat specifically binds to a single base of DNA, whose identity is recognized by amino acids at positions 12 and 13 of the repeat. These two amino acids are called repeat variable residues (RVDs). Co-crystal structure analysis of TALE-DNA binding domain bound to their cognate sites indicates that these two RVDs fall into the DNA major groove. The residue at position 13 makes a base-specific contact to the DNA whereas residues at positions 12 and 8 of the same repeat interact with each other, possibly stabilizing the formation of binding structure (Deng et al., 2012; Mak et al., 2012). There are four repeats that contain the RVDs NN, NI, HD, and NG for recognition of guanine, adenine, cytosine and thymine respectively. It is this simple and predictable 'protein-DNA code' that makes TALENs a preferable choice for targeted genome editing. Indeed, application of TALENs has been shown to significantly enhance HR by > 1000 fold thus facilitating gene targeting (Sun et al., 2012).

The adeno-associated virus integration site 1 (AAVS1) locus refers to the region near the first exon and intron of the *PPP1R12C* (protein phosphatase 1, regulatory subunit 12C) gene on chromosome 19, which is ubiquitously expressed. It is considered a "safe harbor" based on the observation that non-pathogenic adeno-associated virus 2 (AAV2) integrates at this

site. Monoallelic disruption of the *PPP1R12C* gene does not appear to have an adverse effect on the targeted cells (Ogata et al., 2003; Smith et al., 2008; Zou et al., 2011). The AAVS1 site has an open chromatin conformation structure since it presents a DNase I-hypersensitive site (Lamartina et al., 2000). This structure allows trans-acting factors access to perform recombination, transcription, replication, and chromosome segregation (Gross and Garrard, 1988). The open chromatin structure at the AAVS1 site is also associated with cis-acting insulators. Insulators are DNA structures that functionally maintain the gene expression by direct block of enhancers from affecting different promoter domains, thus acting as boundaries to the surrounding heterochromatin that silences the genes located within (Burgess-Beusse et al., 2002). An insulator-like structure has been described at the AAVS1 site (Ogata et al., 2003). Indeed, transgenes integrated in the AAVS1 site show stable and long term expression in a variety of cell types including hESCs and hiPSCs (Hockemeyer et al., 2011; Smith et al., 2008). For example, AAVS1-EGFP expression in hESCs has been shown by both us and other investigators to be robust and persistent in long-term cell cultures. After lineage differentiation, more than 90% of the differentiated cells still express EGFP and are able to maintain fluorescence even after in vivo transplanting into mice infraction heart for at least seven weeks (Smith et al., 2008). Thus, the AAVS1 locus serves as a useful site for generation of fluorescent reporter cell lines in hiPSCs.

Critical Parameters and Troubleshooting

AAVS1-TALENs activity—To integrate EGFP donor in AAVS1 locus in hiPSCs, designed TALENs must efficiently cleave the targeted AAVS1 site to create DSB. Our AAVS1-TALENs target the same DNA sequence described in the previous report (Hockemeyer et al., 2011), but are encoded by a different TALE structure. The activities of these customized TALENs targeting the endogenous AAVS1 site will have an essential impact on efficiency of following transgene integration and need to be first determined. That is why we have described NHEJ assay as a first protocol because it measures TALEN cutting activity at endogenous locus. We have chosen human embryonic 293T cells as cell source since they are easy to culture and have much higher nucleofection efficiency (> 90%) relative to that of hiPSCs (10-50 %). Based on our experiences using this protocol, cutting efficiency of TALENs should be at least 10 – 30% in 293T, which will give a reasonable number of targeted colonies in hiPSCs. NHEJ can be also done in other human cells lines such as K562 or hiPSCs, but results in different efficiencies (higher in K562 and lower in hiPSCs).

Nucleofection efficiency—By combination of Nucleofector™ Programs and cell type-specific transfection solutions developed by Lonza, Nucleofection™ system provides a convenient and non-virus method to deliver nucleic acids such as plasmid into the nucleus directly and efficiently. This system, in our hand, has a very high cell survival (at least 90%) after 2 days of nucleofection in hiPSCs. However, the transfection efficiency is around 10 – 50% depending on the hiPSC lines. In order to increase nucleofection efficiency, we recommend working out optimal conditions using pMaxGFP control vector included in the Kit and your available hiPSC lines, adjusting cell numbers, amounts of plasmids and

Neleofector™ parameters before targeting experiments. Our experiences suggest that >30% nucleofection efficiency can significantly help successful targeting.

Dosage of drug selection—After 48 hr following the nucleofection, the EGFP and puromycin-resistant genes contained in the EGFP donor are expressed in most of the transfected cells and positive selection should begin. The purpose of application of puromycin in cell cultures is to selectively allow the cells with targeted integration to survive since random integration-only is unlikely to drive SA-2A-linked puromycin-resistant gene expression. Theoretically, a dosage that can rapidly kill all but puromycin-resistant cells would be good enough to obtain targeted colonies. However, the dosage usually in such condition is high and too harsh for hiPSC cell cultures, resulting in rapid differentiation without colony formation. To minimize the risk of differentiation, one should first determine killing curve using same culture system as real targeting experiment but without delivering donor or TALENs. Based on killing curve, one should select low dosage of puromycin at the beginning, then gradually increase dosage according to culture conditions of colony formation and differentiation. The selection period should be around 10 days, during which all transient transgene expressed cells are being killed, only integrated cells will survive. At this time point, the colonies should be big enough and are ready to be picked.

Anticipated Results

As we described above, generation of EGFP reporter in AAVS1 site in hiPSCs involves many steps and factors. These include activity of TALENs, EGFP donor, nucleofection efficiency of given hiPSC, drug selection processes, picking and expanding clones, PCR screening and Southern confirmation of EGFP donor insertion without random integration, and finally characterization of the targeted reporter lines. For example, low activity of TALENs will reduce the chance of the homologous recombination. But increasing nucleofection efficiency elevates chance of more cells to get targeted, thus compensating low activity of TALENs. Thus, in addition to pay attention of each step and procedure, one should also consider whole experimental design. Our experiences suggest picking 10-15 clones after puromycin selection and at least 90% of clones were positive by PCR screening, around 30-50% clones are AAVS1-targeted without random insertion (fig. 4).

Time Considerations

Once the AAVS1-TALENs and EGFP donor vectors are obtained, it will take a minimum of 3.5 months to generate and validate a reporter knock-in hiPSC line. This estimation is based on the followings: 2 weeks for NHJE assay, 2 months for targeting, selection and clone expansion, and 1 month for PCR and Southern blot validation. However, it could take several more time if a correctly targeted hiPSC clone is not obtained from the first nucleofection trial. Therefore we recommend repeating targeting experiments while screening the clones from the first round of experiment, until the desired clones are genetically confirmed. If new donor or TALENs need to be constructed, it will take additional time to validate the new constructs and generate reporter hiPSC lines.

Acknowledgments

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Fig. 1.
An outline of procedures to generate EGFP reporter hiPSC line at AAVS1 safe harbor.

Fig. 2. Overview of T7E1 NHEJ assay

AAVS1-TALENs, which are drawn at the top of panel bound to genomic DNA (straight double lines), cleave their targets, resulting in double strand break indicated as a small gap. Since no donor is applied, cellular DNA repair machinery yields minor mutations (jagged double line) through NHEJ pathway. The genomic DNA is extracted. The NHEJ-induced mutations are amplified by PCR. Re-annealing of mutated and wild-type sequence results in mismatched heteroduplexes. This reannealed mixture is digested with T7E1, an enzyme selectively cleaves mismatch DNA. The uncleaved and cleaved products are resolved by agarose gel.

Fig. 3. Calculation of AAVS1-TALENs cutting efficiency

A. Example of 1% agarose gel image of NHEJ assay. C, a control sample without application of TALENs; T, application of a pair of AAVS1-TALENs. **B.** A density profile of T lane analyzed using ChemiDoc™ XRS⁺ with image Lab™ software. The volume of each band is used to calculate fraction as indicated. % cutting efficiency = $100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$.

Figure 4. PCR screening and Southern blot analysis

A. A schematic illustration of AAVS1 gene targeting with AAVS1-CAG-EGFP (a.k.a. AAV-CAGGS-EGFP) donor. Puro expression is driven by endogenous promoter of PPP1R12C gene through a splicing acceptor (SA) linked self-cleaving 2A peptide. EGFP expression is driven by CAG (CAGGS) promoter. 5'F/5'R primers were used for 5' junction PCR screening. The 5' junction PCR amplifies a fragment from intron 1 of PPP1R12C gene, just upstream of left homology arm (AAVS1-LA), to the puro-resistant element of the donor with a size of 1.03 kb. The internal AAVS1 probe (5'pb) is located inside the left homology

arm AAVS1-LA with a size of 705 bp, therefore can recognize wild-type (6.5 kb), targeted integration (3.8 kb) and random integration (other sizes) with SphI enzyme digestion. **B.** Shown are PCR screening results for selected clones. All clones are also EGFP positive. **C.** Southern blot analysis demonstrates that 56% (5/9) clones (red number) are integrated without random insertion. WT, wild type; TI, targeted integration. After SphI digestion, the 5'pb detects WT allele as a 6.5 kb band, and TI as a 3.8 kb band due to the introduction of a SphI site in puro-resistant element.