Methods in Molecular Biology (2015) 1307: 173–190
DOI 10.1007/7651_2014_73

Published online: 11 March 2014

Genetic Modification in Human Pluripotent Stem Cells by Homologous Recombination and CRISPR/Cas9 System

Haipeng Xue, Jianbo Wu, Shenglan Li, Mahendra S. Rao, and Ying Liu

Abstract

Genetic modification is an indispensable tool to study gene function in normal development and disease. The recent breakthrough of creating human induced pluripotent stem cells (iPSCs) by defined factors (Takahashi et al., Cell 131:861–872, 2007) provides a renewable source of patient autologous cells that not only retain identical genetic information but also give rise to many cell types of the body including neurons and glia. Meanwhile, the rapid advancement of genome modification tools such as gene targeting by homologous recombination (Capecchi, Nat Rev Genet 6:507–512, 2005) and genome editing tools such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system, TALENs (Transcription activator-like effector nucleases), and ZFNs (Zinc finger nucleases) (Wang et al., Cell 153:910–918, 2013; Mali et al., Science 339:823–826, 2013; Hwang et al., Nat Biotechnol 31:227–229, 2013; Friedland et al., Nat Methods 10(8):741–743, 2013; DiCarlo et al., Nucleic Acids Res 41:4336–4343, 2013; Cong et al., Science 339:819–823, 2013) has greatly accelerated the development of human genome manipulation at the molecular level. This chapter describes the protocols for making neural lineage reporter lines using homologous recombination and the CRISPR/Cas system-mediated genome editing, including construction of targeting vectors, guide RNAs, transfection into hPSCs, and selection and verification of successfully targeted clones. This method can be applied to various needs of hPSC genetic engineering at high efficiency and high reliability.

Keywords: Gene targeting, Reporter cell line, Genetic engineering, Human induced pluripotent stem cells, Genome editing, Homologous recombination, ZFN, CRISPR, Cas, TALEN

1 Introduction

Genetic modification is an indispensable tool to study gene function in normal development and disease. The recent breakthrough of creating human induced pluripotent stem cells by defined factors (1) and human embryonic stem cells (collectively called hPSCs) provides a renewable source of patient autologous cells that not only retain identical genetic information but also give rise to many cell types of the body including neurons and glia, two major cell types in the central nervous system (CNS). Meanwhile, the rapid advancement of genome modification tools such as gene targeting by homologous recombination (2) and genome editing tools including CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system, TALENs

(Transcription activator-like effector nucleases), and ZFNs (Zinc finger nucleases) (3–8) has greatly accelerated the development of human genome manipulation at the molecular level. This chapter describes the protocols of making neural lineage reporter lines using homologous recombination and CRISPR/Cas system-mediated genome editing, including construction of targeting vectors and guide RNA vectors, transfection into hPSCs, and selection and verification of successfully targeted clones. The reporter proteins (fluorescent proteins, drug-resistant cassettes or other tags) are driven by the endogenous promoters and therefore recapitulate the endogenous gene expression both spatially and temporally. This method can be applied to various needs of hPSC genetic engineering at high efficiency and high reliability. This protocol has been successfully implemented in targeting multiple hPSC lines including iPSC lines derived from Down syndrome patients, amyotrophic lateral sclerosis (ALS) patients, and healthy individuals, as well as human embryonic stem cell (hESC) lines BG01, BG01V, and WA09 (H9), for a variety of purposes. Using this protocol, we have created multiple neural lineage-specific hPSC reporters including HB9-GFP, OLIG2-GFP, NEUROG2-mCherry-hygromycin, and SOX1-GFP-neomycin knockin lines, and have performed gene correction experiments for SOD1 point mutations in ALS patient iPSCs. In addition, using the same strategy, we have generated safe harbor targeting and floxing in hPSCs, a system that allows for subsequent recombinase-mediated cassette exchange (RMCE) and other complicated genetic modifications in hPSCs.

2 Materials

2.1 Targeting Vector Components

2.1.1 Vector Collection

1. pStart-K (Addgene, cat. no. 20346).
2. pWS-TK6 (Addgene, cat. no. 20350).
3. pKD3 (Addgene, cat. no. 45604).
4. pKD46 (The Coli Genetic Stock Center, CGSC cat. no. 7634).
5. EGFP cassette.
6. IRES-mCherry-IRES-Hygromycin cassette.
7. PGK-neo-bpA sequence (Addgene, cat. no. 13442).
8. Human BAC clones of target genes (https://bacpac.chori.org).

2.1.2 CRISPR/Cas9 System Vector Collection

1. JDS246 (Cas9-003), Mammalian codon-optimized *streptococcus pyogenes* Cas9-3X Flag (Addgene, cat. no. 43861).
2. MLM3636, Human-gRNA-Expression Vector with U6 promoter (Addgene, cat. no. 43860).
3. gRNA design: http://zifit.partners.org/ZiFiT/

4. Off-target prediction tool: http://eendb.zfgenetics.org/casot/download.php
5. gRNA Primer synthesis: Sigma.

2.1.3 Molecular Biology Reagents

1. Competent cells: One shot Top10 Electrocomp *E. coli* (Life Technologies, cat. no. C4040-50).
2. AccuPrime Pfx SupperMix (Life Technologies, cat. no. 12344–040).
3. Restriction enzymes (NEB and Life Technologies).
4. Zymoclean Gel DNA Recovery Kit (Zymo Research, cat. no. D4007).

2.2 Cell Culture

1. Stempro hPSC SFM medium (Life Technologies, cat. no. A1000701).
2. Essential 8 medium (Life Technologies, cat. no. A14666SA).
3. Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium (Life Technologies, cat. no. A12856-01).
4. D-MEM/F12 with Glutamax (Life Technologies, cat. no. 10565018).
5. D-MEM with Glutamax (Life Technologies, cat. no. 10566040).
6. Fetal Bovine Serum-ES cell qualified (Life Technologies, cat. no. 10439).
7. Knockout serum replacement (Life Technologies, cat. no. 10828010).
8. 2-Mercaptoethanol 1,000× (Life Technologies, cat. no. 21985023).
9. Non-Essential Amino Acid (Life Technologies, cat. no. 11140050).
10. Stempro Accutase (Life Technologies, cat. no. A1110501).
11. Dispase (Life Technologies, cat. no. 17105–041).
12. 0.25 % Trypsin–EDTA solution (Life Technologies, cat. no. 25200–056).
13. Geltrex (Life Technologies cat. no. 12760–013).
14. ROCK inhibitor (Y-27632, Millipore, cat. no. SCM075).
15. SMC4 reagent (BD, cat. no. 354357).
16. Neomycin-resistant MEF (Millipore, cat. no. PMEF-NL).
17. Hygromycin-resistant MEF (Millipore, cat. no. PMEF-HL).
18. Neomycin-, hygromycin-, and puromycin-resistant MEF can be made from DR-4 mouse strain as multiple drug-resistant MEFs (Jackson laboratory, Tg DR4 1Jae/J, Stock Number: 003208).

19. G418 (Geneticin, Life Technologies, cat. no. 11811).
20. Hygromycin B (Life Technologies, cat. no. 10687010).
21. FIAU (Fialuridine, 1-(2-Deoxy-2-fluoro-ß-D-arabinofuranosyl)-5-iodouracil, Moravek Biochemicals and Radiochemicals, cat. no. M251).
22. *hPSC cell lines*: ND2.0, ND1.4, house reprogrammed iPSCs.

2.3 Electroporation Equipment and Reagents

1. Electroporator: Gene Pulser Xcell (Bio-Rad).
2. 0.4 cm electroporation cuvette (Bio-Rad, cat. no. 165–2088).

2.4 Genomic PCR and Southern Blot

1. DIG-High prime DNA labeling and detection starter kit II (Roche, cat. no. 11585614910).
2. Hybridization denature solution (VWR, cat. no. 82021–478).
3. PCR DIG probe synthesis kit (Roche, cat. no. 11636090910).
4. DIG wash set (Roche, cat. no. 11585762001).
5. Anti-Digoxigenin (DIG)-AP (Roche, cat. no. 11093274910).
6. CSPD chemiluminescence system (Roche, cat. no. 11755633001)
7. DIG wash and block buffer set (Roche, cat. no. 11585762001).
8. 50× TAE buffer (Life Technologies, cat. no. 24710030)

   Blotting buffer (25 mM Tris pH 7.4, 0.15 M NaCl, 0.1 % Tween20).
9. Hoefer Ultraviolet Crosslinker (Fisher Scientific, cat. no. 03-500-308).
10. Spermidine (Fisher, cat. no. AC13274-0010).
11. Tris–HCl 2 M, pH 7.5 (VWR, cat. no. 200064–506).
12. Denville Scientific blue bio film 8 × 10 (Fisher, cat. no. nc9550782).
13. DNA molecular weight marker II, DIG-labeled (VWR, cat. no. 11218590910).
14. Amersham Blotting membrane Hybond-N+ (Roche, cat. no. 95038–400).
15. Pyrex glass drying tray (Fisher, cat. no. 15-242A).
16. Kimberly-Clark C-fold paper towels (Fisher, cat. no. 06-666-32B).
17. Whatman 3MM paper, 26 × 41 (Fisher, cat. no. 05-713-336).
18. Hybridization bag (Roche, cat. no. 11666649001).
19. Hybridization tubes (Fisher, cat. no. 13-247-300).
20. Hybridization oven rotisserie Shake “n” Stack (Fisher, cat. no. HBMSOV14110).

3 Methods

3.1 Vector Construction

3.1.1 General Suggestions

Vector construction protocol follows the published instructions (9–11). Briefly, the targeting vectors are designed to have a floxed positive selection (usually is an antibiotic resistant) cassette and a negative selection (thymidine kinase, TK, or diphtheria toxin A, DTA (12)) cassette, which is located right outside of the 3′ homology arm. To enhance negative selection, an additional copy of DTA can be added upstream of the 5′ homology arm. Reporter genes include a variety of gene cassettes, with different flavors of fluorescent proteins (e.g., green or red fluorescent proteins), lacz, drug selection, etc. The purpose of using gene targeting is to target the reporters or dual reporters precisely to the endogenous locus. To prevent the interference of fusion reporter proteins with the expression of gene of interest (GOI) and to preserve both copies of GOI and avoid haploinsufficiency, we choose to construct targeting vectors with GOI tagged by a fluorescent protein and a drug selection, which are connected to the GOI sequentially by an IRES (internal ribosome entry site) cassette. Alternatively, the self-processing viral 2A peptides, F2A (from the foot-and-mouth disease virus), E2A (from the equine rhinitis A virus), T2A (from the *Thosea asigna* virus), or P2A (from the porcine teschovirus) (13, 14) can be used in the place of IRES. These strategies allow for the concurrent expression of fluorescent proteins and drug selections under the promoter of the endogenous GOIs. An example is shown in Fig. 1 for constructing targeting vector for human SOX1 gene, tagged by EGFP and neomycin resistance cassette.

Briefly, a human BAC clone containing the GOI is purchased from Life Technologies or Children's Hospital and Research Center at Oakland (https://bacpac.chori.org). The clone is verified by PCR amplification of the gene. The targeting vector is constructed in DH5α using recombineering and multisite gateway as described (9, 10). The resultant constructs have the following format: 5′ homo arm-endogenous GOI-IRES-Fluor protein-IRES-Drug selection-3′ homo arm-TK. After linearization, targeting vectors are electroporated into hPSCs.

3.1.2 Design and Vector Construction for Cas9 and gRNA for GOI

Recently we have quickly implemented the CRISPR/Cas (3–8) system in the lab, by which one-step concurrent mutation for multiple genes has been reported in the mouse (3). We have designed and obtained multiple guide RNAs (gRNAs) (Table 1 shows an example of gRNAs designed for genetic correction of SOD1 protein at the 139th amino acid of N→K) using Golden Gate (15–21) cloning. To precisely target to GOI, we have applied the following rules in CRISPR designs and practice. (a) Improve specificity and minimize off-target mutagenesis by choosing unique "seed sequence" with low GC and high AT content, and use the

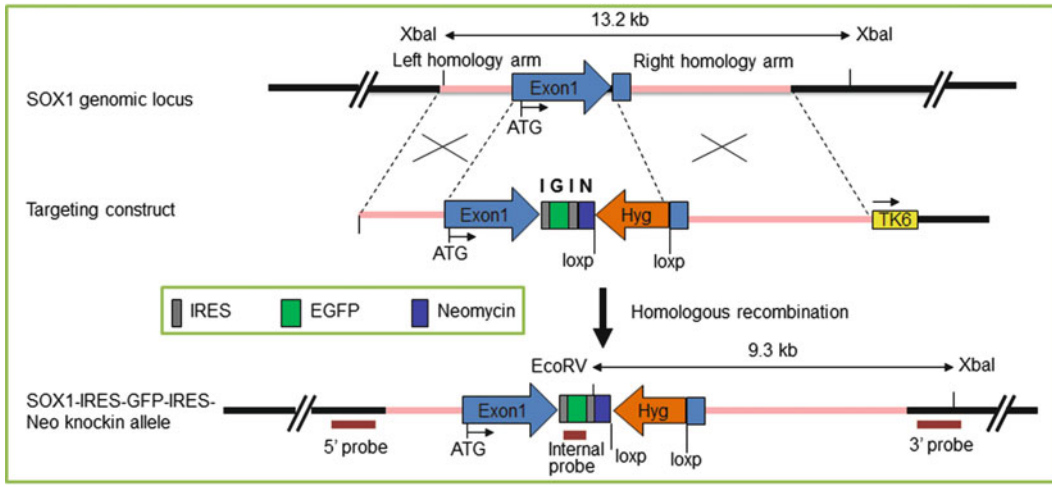

Fig. 1 Targeting vector design for creating human SOX1 gene knockin reporter hPSC line. SOX1 genomic sequence is tagged by dual reporter cassettes, EGFP and neomycin resistance, both of which are connected by IRES and therefore driven by the endogenous SOX1 promoter. EGFP and neomycin can both be used for selection and purification of SOX1 expressing cells. Please note that Hygromycin resistance and TK6 cassettes are included for positive and negative selections, respectively, during isolation of targeted hPSC clones

Table 1
Guide RNA (gRNA) sequence for genetic correction of SOD1 N139K mutation

Name	Sequence 5′→3′
SOD1_N139K_gRNA1	GCTGGAAGTCGTTTGGCTTGTGG
SOD1_N139K_gRNA2	GCAGATGACTTGGGCAAAGGTGG
SOD1_N139K_gRNA3	GGGCCTCAGACTACATCCAAGGG

minimal combined amount of gRNA and Cas9 plasmids. (b) Optimize the ratio of hiPSC number and the amount of total plasmid DNA of gRNA and Cas9. Based on the literature (3) and our own experience, for 1×10^6 hiPSCs, we will use 5 μg gRNA expression vector and 5 μg Cas9 expression vector total. (c) Rank gRNAs for each gene based on indel % from SURVEYOR assay (Fig. 2), and choose the gRNAs with "medium" efficiencies to avoid off-target events. (d) Monitor the number of targeted copies by Southern blot. Potential off-target sites can be predicted using an online open source tool http://eendb.zfgenetics.org/casot/download.php, which is a Perl-based program (http://www.perl.org/get.html). An example of potential off-target sites for the gRNAs designed for genetic correction of SOD1 mutation (N139K) is listed in Table 2.

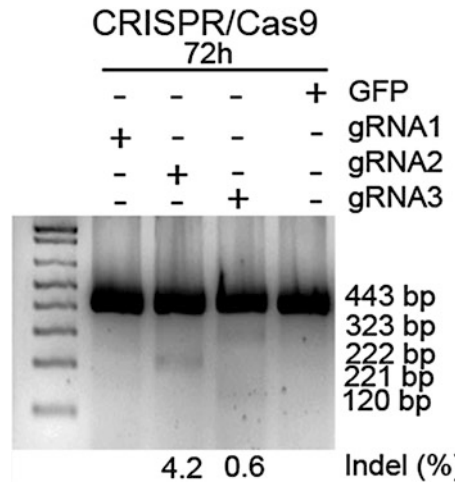

Fig. 2 SURVEYOR assay of guide RNA (gRNA)-mediated cleavage in 293 cells by the CRISPR/Cas9 system. The gRNAs are designed to facilitate gene correction of a point mutation of AAC→AAA that changes the 139th aa from N(Asn) to K (Lys) in the human SOD1 gene in Amyotrophic Lateral Sclerosis (ALS) patient iPSCs. gRNA2 and gRNA3, which result an indel of ~4 % and 0.6 % respectively, can be used for gene correction in ALS SOD1 N139K patient iPSCs

3.2 Transfection of hPSC to Create Homologous Recombinants

3.2.1 Culture hPSCs on MEF

Single cells are needed for electroporation to generate homologous recombinants. hPSCs cultured on mouse embryonic fibroblasts (MEFs) can be adapted to feeder-free MEF-conditioned medium, and passaged in a single cell fashion for electroporations. Commercially available, chemically defined media such as Stempro SFM (Life Technologies) and mTesR (Stem Cell Technologies) can also be used for subculture. Usually it takes 2–3 days for the cells to get fully adapted to a new type of medium; therefore, constantly changing culture medium type is not recommended. After electroporation, cell recovery is improved if they are plated onto MEF in MEF-conditioned medium with ROCK inhibitor or SMC-4 (Fig. 3). Therefore maintaining the cells on MEF or in MEF-CM is optimal for this particular set of electroporation experiments. If chemically defined medium other than MEF-CM is used, give the cells an additional 2–3 days for adaptation. Essential 8 medium (Life Technologies), another very popular hPSC medium, does not support single cell passage; therefore, it can only be used for intermediate steps of subculture.

1. MEFs are prepared from E13.5 mouse embryos and cells from passage 1 saved in the liquid nitrogen storage. Cells of up to passage 3 can be inactivated by irradiation which can then be used as feeders to support hPSC growth. The irradiation dosage is 8000 Rad (cGy). Inactivated MEFs can be aliquoted as desired and stored in liquid nitrogen.
2. The day prior to thawing the hPSCs, seed inactivated MEF dishes at a density of 3×10^4 cells/cm^2 in MEF medium.

Table 2
Potential off-target sites for guide RNAs (gRNA2 and gRNA3) designed for gene correction of SOD1 N139K by gene targeting

gRNA	Coordinate (chromosome: location:strand)	Sequence	Mm (seed)	Mm (nonseed)	Exon information
gRNA2	8:126964170–126964193:–	cCAGATGA_CTTGGG CAAAGa-TGGA	1	1	SOD1P3
gRNA2	14:69520998–69521021:+	cCAGATGc_CTTGGGgAc AGG-AGGA	2	2	DCAF5
gRNA2	15:62360764–62360787:–	GCAGAaGt_CcTGGtCA AAGG-AGGC	2	2	C2CD4A
gRNA2	16:25157949–25157972:–	GCtGtTGA_CTTtGGC AgAGG-CGGC	2	2	RP11-266L9.2
gRNA2	19:53594799–53594822:+	GCgGggGA_CTTGGGg AAAGG-GGGC	1	3	ZNF160
gRNA2	2:132238074–132238097:–	tgAGATGA_CTgGGGC AtAGG-TGGC	2	2	TUBA3D
gRNA2	7:27788177–27788200:–	aaAGATGA_CaTGGG CAAAGt-TGGA	2	2	TAX1BP1
gRNA2	7:96653811–96653834:+	GCtGAaGA_CTcGGG CAAAGt-TGGC	2	2	DLX5
gRNA3	16:19098321–19098344:+	aGGCaTCA_GACaA CATCCAA-GGGG	1	2	RP11-626G11.4

Notes: *Mm* mismatch
Generally, if the combined number of Mms within seed and nonseed sequence is >4, off-target activities are unlikely. If the number of Mms within the seed sequence is <2, off-target events need to be monitored and examined
Mismatched bases are in lower case

3. Take hPSCs (~2 × 10^6 cells/vial) from liquid nitrogen storage to 37 °C water bath immediately. Transfer all contents from the vial to a 50 mL conical tube and add prewarmed hPSC growth medium dropwise up to 10 mL.
4. Spin at 200 × *g* for 4 min at room temperature and aspirate the supernatant.
5. Resuspend cells in 1 mL of hPSC growth medium and plate onto the MEF dish prepared earlier (step 2).
6. When cells are ready to be passaged, aspirate medium and add 3 mL dispase solution per 100 mm dish.
7. Incubate until the edges of colonies start to curl up.
8. Gently triturate the clumps using a 5 mL serological pipette and transfer the clumps onto a 15 mL conical tube.

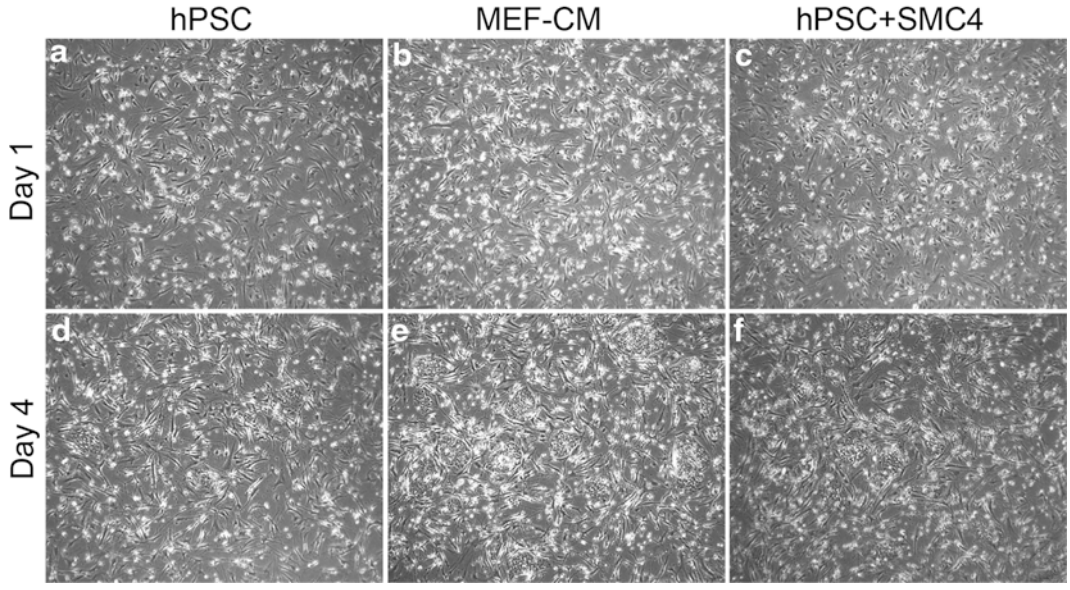

Fig. 3 SMC4 improves single cell survival after electroporation. Equal number of hPSCs were plated after electroporation in hPSC medium (**a**, **d**), or MEF-conditioned hPSC medium (MEF-CM, **b**, **e**), or hPSC medium supplemented with SMC4 reagent (SMC4, **c**, **f**). Significantly higher number of cells and colonies appeared in MEF-CM and SMC4 groups at both day 1 and 4 postelectroporation

9. Spin at 200 × *g* for 4 min at room temperature and aspirate the supernatant.
10. Gently aspirate medium and resuspend the cells in hPSC growth medium.
11. Transfer the cell clumps onto new MEF dishes at a ratio of 1:4, every 4–5 days.

3.2.2 Electroporation

Transfer of hPSCs onto Geltrex-Coated Dishes for Feeder-Free Culture

1. Preparation of Geltrex-coated dishes. Thaw a whole bottle of Geltrex at 4 °C for 3 h or overnight to prevent polymerization (*see* **Note 1**).
2. Add equal volume of cold DMEM/F12 to make 100× stock solution and store desired aliquots at −20°C.
3. Before coating plates, thaw aliquots at 4 °C or on ice. Add appropriate volume of DMEM/F12 to make 1× solution and add to culture dishes to completely cover the surface area (e.g., 2–3 mL/60 mm dish), and incubate at room temperature for 1 h.
4. Coated dishes can be used immediately after coating or stored at 4 °C for 2 weeks.
5. Avoid drying the dishes and remove coating solution immediately before use.

6. Passage of hPSCs onto Geltrex-coated dishes: Harvest cells following Section 3.2.1, steps 6–11.
7. Instead of spinning down the cell pellets, let the cells settle to the bottom of the tube by allowing the tube to sit in the tissue culture hood for 5–10 min (*see* **Note 2**).
8. Remove supernatant, and aliquot cells at 1:4 ratio onto Geltrex-coated dishes with MEF-CM supplemented with fresh bFGF (12 ng/mL).
9. When cells reach ~80 % confluency, seed the hPSC onto five 10-cm, Geltrex-coated dishes with CM with fresh bFGF (12 ng/mL). When cells reach ~80% confluency, they can be used for electroporation.

3.2.3 Electroporation, Antibiotic Selection, and Isolation of Clones

1. One day before electroporation, prepare six 10-cm *drug*-resistant MEF dishes (*see* **Note 3**).
2. Electroporation. Prewarm 12 mL of MEF-CM in a 15 mL tube at 37 °C.
3. Aspirate medium from Geltrex-coated plate, add 2.5 mL accutase per 10 cm dish (*see* **Note 4**) before adding to dishes. Leave it at room temperature for 3–5 min until the cells dislodge.
4. Collect cells by adding 5 mL DMEM/F12 medium, wash once, and collect everything into a 15 mL conical tube. Take an aliquot of cells for counting.
5. Aliquot 1×10^7 cells to a 15 mL conical tube containing 10 mL DPBS, centrifuge at $200 \times g$ for 5 min. Remove supernatant as much as possible. Resuspend cells with 700 μL DPBS (the total final volume is 800 μL).
6. Add 50 μg linearized targeting vectors and mix well. Transfer the cell mixture to a 0.4 cm cuvette.
7. Alternatively, if CRISPR/Cas9 system is used, prepare 1×10^6 cells, and electroporate 5 μg Cas9 vector, 5 μg gRNA vector, and 5 μg targeting (donor) vector. Proceed to step 8 (*see* **Note 5**).
8. Electroporate cells using the following parameters: 250 V, 250 μF, ∞Ω. Immediately transfer the cells to a 15 mL conical tube containing 12 mL MEF-CM. Mix the cells, put it at room temperature for 5 min and mix the cells gently once per minute by tapping the tube during incubation.
9. Transfer the cells to six 10-cm *drug*-resistant MEF dishes containing 8 mL MEF-CM. Add FGF so that the final FGF concentration is 12 ng/mL. Return the cells to the incubator at 37 °C with 5 % CO_2.

10. To enhance single cell survival, 10 μm ROCK inhibitor or SMC-4 can be added for the first 24 h post electroporation (*see* **Note 6**).
11. Change medium everyday for day 1–3 with 10 mL MEF-CM containing 12 ng/mL bFGF.
12. From day 4 to 8, change medium everyday with 10 mL freshly made hPSC medium containing 12 ng/mL bFGF.
13. Individual colonies start to appear around day 3–5. Usually about 200 colonies can be seen by day 7 per 100 mm dish. At this point, start positive selection.
14. Seven days later, *start negative selection* using FIAU (200 nM) together with positive selection in the hPSC medium. Negative selection may last for 2–3 weeks.
15. Culture the cells under both positive and negative selections for 21 days.
16. When individual colonies are visible to the naked eye, but are not merging with neighboring colonies, they are ready to be isolated. Manually isolate single clones using a glass tool made from a Pasteur pipette (*see* **Note 7**) or a 25 G × 1/2 in. syringe needle. Carefully grid and dislodge the colony using the tool, and use a p200 pipette with a 200 μL tip to transfer the dissected clumps into one well of a 24 well plate. Make sure to completely remove all dislodged pieces before dissecting the next clone to avoid cross clonal contamination (*see* **Note 8**).
17. Continue to culture and expand individual clones under positive selection in hPSC medium. Save cell pellets (2–5 × 10^5 cells) to extract genomic DNA for Southern blot analysis to identify homologous recombinants (continue to Section 3.3).
18. For positive clones, split cells every week at a 1:2 ratio. When cells reach to ~5 × 10^6 (~two 60 mm dishes), freeze and stock 2–3 vials per clone and continue culturing. This takes 2–3 weeks (*see* **Note 9**)

3.3 Identification of Homologous Recombinants

Clones obtained by positive and negative selection will be verified by Southern blot analysis using a nonradioactive protocol with Digoxigenin (DIG)-labeled probe and detection system.

3.3.1 Probe Design

Prepare probes using the DIG-labeling and detection system kit from Roche. Genomic DNA is used as a template to first amplify a nonlabeled probe sequence. 5′-, 3′- and internal probes are designed based on the GOI and the targeting homology arms. The PCR product for probes is then cloned into a TA vector and will be used as a template for making DIG-labeled probes. PCR cycling condition is 95 °C 2 min, 30 cycles of 95 °C 30s → 60 °C 30s → 72 °C 40 s, with final extension at 72 °C for 7 min.

Table 3
Design of Southern blot probes for SOX1-IRES-EGFP-IRES-NEO gene targeting

Probes	Size of probe (bp)	Restriction enzymes	WT band (kb)	Targeted band (kb)
5′	804	EcoRV + XhoI	7.7	9.3
3′	530	EcoRV + XbaI	13.2	9.3
Internal	632	EcoRV + XhoI	7.7	9.3

Notes: Primers (5′→3′) for making 5′ probe: GGTGAGCCCCTACTCCAAAGCT and ATGAGGGGCAAAGAGG-CAGC; 3′ probe: GAAATTACAAAACCAGCTTCCCAGG and TGTACCTCCTCTGCAAAACCTTCCT; internal probe: ATGGTGAGCAAGGGCGAGGA and TTGGGGTCTTTGCTCAGGGC

Table 4
Preparation of DIG-labeled probes

Order	PCR reaction tube	GOI DIG-probe	GOI control	tPA DIG-control	tPA control
1	H_2O, PCR grade	33.25 μL	33.25 μL	29.25	29.25
2	10× PCR buffer	5 μL	5 μL	5 μL	5 μL
3	PCR DIG mix	5 μL	–	5 μL	–
4	dNTP	–	5 μL	–	5 μL
5	10× PCR Primer mix[a]	5 μL	5 μL	–	–
5	Control primer	–	–	5 μL	5 μL
6	Enzyme mix	0.75 μL	0.75 μL	0.75 μL	0.75 μL
7	DNA template[b]	1 μL	1 μL	–	–
7	Control template	–	–	5 μL	5 μL

Notes: [a]10× PCR Primer mix is comprised of both forward and reverse primers at a stock concentration of 5 μM, the final concentration of each primer in reaction mix is 1 μM
[b]If genomic DNA is used, then add 1–50 ng of DNA; If plasmid DNA is used, then add 10–100 pg. Adjust volume to 1 μL

Special attention needs to be paid to the final probe sequence. Excessive consecutive thymidine sequence in the template will result in failure of the integration of DIG-dUTP. An example for designing probes for targeting the SOX1 locus is listed in Table 3.

3.3.2 Preparation of the DIG-Labeled Probe

Reaction mix is prepared as shown in Table 4. The ingredients are added to the reaction tubes (kept on ice) in the order indicated in the table. Preparation of reaction mix.

3.3.3 Examination of the Probe

After PCR, 5 μL of DIG-labeled probe is examined by running on a 1 % agarose gel in 1× TAE buffer. Note that DIG-labeled DNA probe migrates slower than nonlabeled DNA. For instance, the probe amplified from the 5-kb control plasmid that contains the cDNA for the human tissue-type plasminogen activator (tPA) is 442 bp, while the DIG-labeled tPA probe migrates to the

Table 5
Restriction enzyme digestion of genomic DNA from hPSC clones

Ingredients	Amount/reaction (μL)
10× RE buffer	2.5
100× BSA	0.5
100 mM Spermidine	1
RE (20 U/μL)	2
H_2O	4
Genomic DNA	15

Notes: *RE* Restriction enzyme
Genomic DNA is adjusted to 300 ng/μL; therefore the total amount is 3–5 μg

500–550 bp position. The 530-bp Olig2 probe migrates to about the 600 bp position once it is DIG-labeled.

3.3.4 Genomic DNA Restriction Enzyme Digestion: Details Below

Genomic DNA (a minimum of 3 μg DNA, from approximately 2–5 × 10^5 cells) is digested using appropriate restriction enzymes as designed. Ingredients are shown in Table 5.

3.3.5 Electrophoresis and Membrane Transfer

1. Make a 0.9 % TAE agarose gel. Load 2 μL DIG-DNA marker. Load the ordinary, nonlabeled 1 kb DNA ladder in an adjacent lane. Load digested DNA and run the gel at 20–25 V overnight (or 100 V for 2 h). Stain the gel by SYBR green and take the gel image with a UV ruler placed on the side. Remove extra gel that is not going to be transferred.
2. Depurinate DNA if the size of the predicted bands is >5 kb. Soak the gel in 0.2 M HCl for 10 min on a horizontal shaking platform or until the blue dye turns yellow color. Rinse gel with sterile ddH_2O.
3. Denature DNA by soaking gel in 0.5 M NaOH, 1.5 M NaCl for 15 min twice, with gentle shaking on a horizontal shaking platform. Rinse gel with sterile ddH_2O.
4. Neutralize DNA in 0.5 M Tris–HCl pH7.5, 1.5 M NaCl for 15 min, twice, with gentle shaking.
5. Equilibrate DNA in transfer buffer 20× SSC (0.3 M sodium citrate, 3 M NaCl) for 15 min, with gentle shaking.
6. Soak Hybond N+ membrane in H_2O and then in 20× SSC for 5 min.
7. Set up transfer as shown in Fig. 4.
8. Crosslink the membrane. Place the membrane with the DNA side facing up on top of a wet 3MM paper (previously soaked in ddH_2O). Place the paper and membrane on the top of

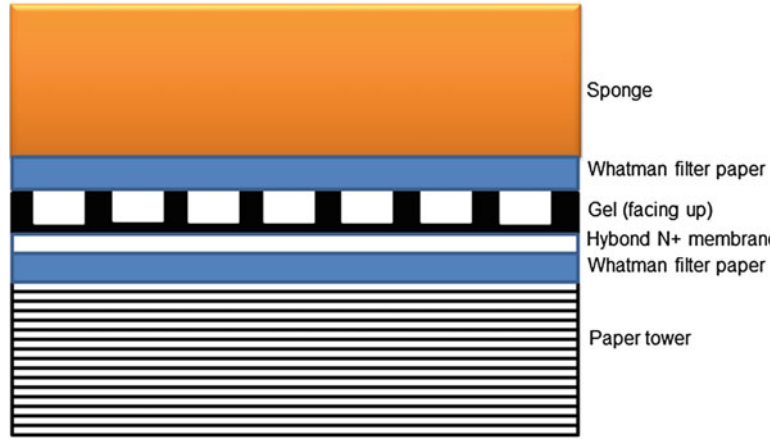

Fig. 4 Scheme for setting up DNA transfer from gel to membrane

XL-1000 UV Crosslinker. Turn on power, press auto crosslink, press start to expose the wet membrane to UV light. Rinse the membrane briefly with ddH_2O, air-dry the membrane for 15 min, and store the dry blot between two sheets of 3MM-paper in a sealed bag at 4 °C. The membrane can be stored for up to 1 month.

3.3.6 Prehybridization and Hybridization

1. *Prehybridize using DIG Easy Hyb* (*see* **Note 10**). Heat 25 mL Hyb-granule solution to 45 °C in a 50 mL sterile tube. Fill hybridization tube with sterile ddH_2O. Place the membrane into hyb tube with the DNA side facing inward. Ensure that the membrane sticks tightly onto the tube wall with no air bubbles. Remove ddH_2O, add 15 mL prewarmed Hyb-granule solution. Incubate at 45 °C for 1 h in hybridization oven with rotation.
2. *Denature the DIG-probe*. Add 20 μL PCR DIG-probe to 50 μL H_2O in a 1.7 mL microcentrifuge tube. Boil the DIG-probe for 10 min and chill on ice for 3 min (*see* **Note 11** for conditions for reused probes).
3. Add the denatured DIG-probe to 10 mL prewarmed Hyb-granule solution. Mix well by gentle inversion to avoid foaming as bubbles may increase background.
4. *Hybridization*. Remove Hyb-granule solution from the Hyb tube (*see* **Note 12**), and replace with 10 mL DIG-probe/Hyb mixture to the Hyb tube with membrane. Incubate at 45 °C overnight with rotation.
5. *Posthybridization stringency washes*. Remove DIG-probe/Hyb mixture (*see* **Note 13**). Fill the Hyb tube with 250 mL Wash buffer I (2× SSC, 0.1 % SDS), wash at 25 °C for 15 min. Repeat the wash once.

6. Fill the tube with 250 mL Wash buffer II (0.5× SSC, 0.1 % SDS), wash at 68 °C for 30 min. Repeat the wash once (*see* **Note 14**).
7. *Immunological detection* (*see* **Note 15**). Remove Wash buffer and replace with 100 mL maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH7.5, 0.3 % Tween 20); gently rotate for 5 min at room temperature.
8. Replace with 120 mL freshly made blocking buffer and gently rotate for 60 min at room temperature (*see* **Note 16**).
9. Replace with 30 mL anti-DIG buffer and gently rotate for 30 min at room temperature (*see* **Note 17**).
10. Replace with 100 mL maleic wash buffer; gently rotate for 15 min at room temperature. Repeat once.
11. Replace with 50 mL Detection buffer (0.1 M Tris pH 9.5, 0.1 M NaCl) and gently rotate for 5 min at room temperature.
12. Place the membrane on a clean plastic sheet (*see* **Note 18**) with the DNA side facing up.
13. Apply 1–2 mL CSPD onto the membrane and cover up with the plastic sheet. Avoid air bubbles.
14. Incubate for 5 min at room temperature.
15. Squeeze out the excess liquid, and seal the plastic sheet with a plastic sealing machine. Incubate for 10 min at 37 °C incubator.
16. Expose to X-ray film for 2 h (at room temperature) to overnight (4 °C).

3.3.7 Blot Stripping and Rehybridization

1. Rinse the membrane with water and wash in stripping buffer (0.2 M NaOH with 0.1 % SDS) at 37 °C for 15 min. Repeat once.
2. Rinse the membrane with 2× SSC for 5 min once.
3. Expose the membrane to X-ray film overnight (as described in Section 3.3.6, step 16) to make sure that no residual signal is present.
4. Proceed to prehybridization and hybridization steps as described in Section 3.3.6 (*see* **Note 19**).

4 Notes

1. When handling Geltrex, keep undiluted Geltrex on ice or in 4 °C at all times.
2. When generate hPSC feeder-free culture from MEF culture, extra care is needed to avoid carrying over MEFs. Letting the pellets settle in the tubes instead of spinning down will largely

eliminate MEFs which are single cells and settle slower than hPSC clumps.

3. The type of drug-resistant MEFs is determined by the vector design for positive selection. Commonly used positive selection cassettes are neomycin, hygromycin, puromycin, and zeocin. Single- or triple-resistant MEFs or mouse strains are available commercially (*see* Materials). Wild type MEFs confer resistance to zeocin at <10 μg/mL, while most unmodified hPSCs will die if cultured in 2 μg/mL zeocin for less than 3 days. Therefore, WT MEFs can be used as feeders for zeocin selection.
4. Accutase should be kept cold at 4 °C.
5. Long-term effects of the Cas9 system in genetic engineering have yet to be comprehensively evaluated.
6. ROCK inhibitor or SMC-4 will only be used for one day (day 1) right after electroporation. Prolonged usage of ROCK inhibitor or SMC-4 might increase the possibility of genetic instability and is not cost-effective. Starting from day 2, regular MEF-CM with 12 ng/mL FGF will be used, and starting from day 8, regular hPSC medium with 12 ng/mL FGF will be used.
7. When isolating individual clones, manual picking is necessary. We have been using 9-in. Pasteur pipettes to make a glass knife, which can grid and cut clones. Alternatively, a p20 pipette tip can be used. Avoid cross clonal contamination. There should be about 180–200 colonies maximum per 10 cm dish.
8. Based on the number of clones, prepare 24 well inactivated MEF plates 1–2 days before planned manual isolation. Usually, at least 50 clones should be isolated. For CRISPR/Cas9 mediated homologous recombination, the targeting efficiency is greatly improved. Positive homologous recombinants should appear in as few as 5–10 colonies. However, because off-target events cannot be completely excluded in CRISPR/Cas9 targeted clones, to guarantee the identification of qualified homologous recombinants, 10–20 colonies should be isolated.
9. It is good to save cells and pellets from 2 to 4 nonhomologous recombinants. These clones can serve as negative controls.
10. Do not dry the membrane at any time. Wear gloves when handling membranes, gels, and reagents.
11. For reused probes, denature at 68 °C for 10 min. Do not boil.
12. Hyb-granule solution can be saved at −20 °C for reuse.
13. DIG-probe/Hyb mixture may be saved at −20 °C for reuse. When reuse, denature the probe at 68 °C for 10 min. Do not boil.

Table 6
Reagent setup for DIG signal detection in Southern blot analysis

Contents	Working buffer	Storage
DIG Hyb granules (power)	Add 32 mL H_2O, stirring at 37 °C for 5 min, and add 32 mL H_2O again	−20°C
10× Blocking solution	For 15 mL 10× blocking buffer, add 15 mL 10× maleic solution and 120 mL H_2O	Freshly made
Anti-DIG solution	For 3 μL Anti-DIG solution, add 30 mL blocking buffer	Stable for 12 h at 4 °C
CSPD Chemiluminescence	Ready-to-use	4 °C

14. The temperature for Wash I and Wash II is different. Wash I is at 25 °C and Wash II is at 68 °C.
15. Detection reagent setup can be found in Table 6.
16. Blocking buffer is made with 15 mL of 10× blocking buffer (which can be *saved at* −20 °C), 15 mL of 10× maleic acid, and 120 mL water. Blocking buffer needs to be made freshly.
17. Anti-DIG buffer is made by adding 3 μL anti-DIG-AP conjugate into 30 mL blocking buffer.
18. The plastic sheet is big enough to cover three times the size the membrane.
19. Stripping and rehybridization only apply to probes that are designed to hybridize to genomic DNAs digested with the same restriction enzyme.

Acknowledgments

This work was supported by the Department of Neurosurgery, Memorial Hermann Foundation-Staman Ogilvie Fund, the Bentsen Stroke Center, at the University of Texas Health Science center at Houston, Robert Packard Center for ALS Research at Johns Hopkins (Grant No. 2000260538), California Institute for Regenerative Medicine (Grant No. RT1-01107-1) to YL, and funds from the National Institutes of Health, Center for Regenerative Medicine to MSR. We thank Dr. J. Zhang for technical assistance.

References

1. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
2. Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6:507–512
3. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153:910–918
4. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA-guided human genome engineering via Cas9. Science 339:823–826
5. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31:227–229
6. Friedland AE, Tzur YB, Esvelt KM, Colaiacovo MP, Church GM, Calarco JA (2013) Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat Methods 10(8):741–743. doi:10.1038/nmeth.2532
7. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res 41:4336–4343
8. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823
9. Wu S, Ying G, Wu Q, Capecchi MR (2008) A protocol for constructing gene targeting vectors: generating knockout mice for the cadherin family and beyond. Nat Protoc 3:1056–1076
10. Xue H, Wu S, Papadeas ST, Spusta S, Swistowska AM, MacArthur CC, Mattson MP, Maragakis NJ, Capecchi MR, Rao MS, Zeng X, Liu Y (2009) A targeted neuroglial reporter line generated by homologous recombination in human embryonic stem cells. Stem Cells 27:1836–1846
11. Liu Y, Jiang P, Deng W (2011) OLIG gene targeting in human pluripotent stem cells for motor neuron and oligodendrocyte differentiation. Nat Protoc 6:640–655
12. Yagi T, Nada S, Watanabe N, Tamemoto H, Kohmura N, Ikawa Y, Aizawa S (1993) A novel negative selection for homologous recombinants using diphtheria toxin A fragment gene. Anal Biochem 214:77–86
13. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA (2004) Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 22:589–594
14. Carey BW, Markoulaki S, Hanna J, Saha K, Gao Q, Mitalipova M, Jaenisch R (2009) Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc Natl Acad Sci U S A 106:157–162
15. Engler C, Gruetzner R, Kandzia R, Marillonnet S (2009) Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One 4:e5553
16. Engler C, Kandzia R, Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS One 3: e3647
17. Engler C, Marillonnet S (2011) Generation of families of construct variants using golden gate shuffling. Methods Mol Biol 729:167–181
18. Thieme F, Engler C, Kandzia R, Marillonnet S (2011) Quick and clean cloning: a ligation-independent cloning strategy for selective cloning of specific PCR products from non-specific mixes. PLoS One 6:e20556
19. Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S (2011) A modular cloning system for standardized assembly of multigene constructs. PLoS One 6:e16765
20. Weber E, Gruetzner R, Werner S, Engler C, Marillonnet S (2011) Assembly of designer TAL effectors by Golden Gate cloning. PLoS One 6:e19722
21. Werner S, Breus O, Symonenko Y, Marillonnet S, Gleba Y (2011) High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. Proc Natl Acad Sci U S A 108:14061–14066