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The iCRISPR platform for rapid genome editing in human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSCs) have the potential to generate all adult cell types, including rare or inaccessible human cell populations, thus providing a unique platform for disease studies. To realize this promise, it is essential to develop methods for efficient genetic manipulations in hPSCs. Established using TALEN (Transcription activator-like effector nuclease) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) systems, the iCRISPR platform supports a variety of genome-engineering approaches with high efficiencies. Here we first describe the establishment of the iCRISPR platform through TALEN-mediated targeting of inducible Cas9 expression cassettes into the *AAVS1* locus. Next, we provide a series of technical procedures for using iCRISPR to achieve one-step knockout of one or multiple gene(s), “scarless” introduction of precise nucleotide alterations, as well as inducible knockout during hPSC differentiation. We present an optimized workflow, as well as guidelines for the selection of CRISPR targeting sequences and the design of single-stranded DNA (ssDNA) homology directed DNA repair templates for the introduction of specific nucleotide alterations. We have successfully used these protocols in four different hPSC lines, including human embryonic stem cells and induced pluripotent stem cells. Once the iCRISPR platform is established, clonal lines with desired genetic modifications can be established in as little as one month. The methods described here enable a wide range of genome-engineering applications in hPSCs, thus providing a valuable resource for the creation of diverse hPSC-based disease models with superior speed and ease.

Keywords

genome engineering; human pluripotent stem cells (hPSCs); human embryonic stem cells (hESCs); CRISPR/Cas; TALENs; gene targeting

1. Introduction

Functional analysis of sequence variants affecting diverse human traits, including disease susceptibility, is key to understanding human biology and disease mechanisms. With their

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unlimited self-renewal capacity and the potential to generate all adult cell types, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), offer an ideal platform for biological and disease studies (Pattanayak et al., 2013; Zhu and Huangfu, 2013). To meet this goal, it is mandatory to develop efficient methods for genome engineering in hPSCs.

The development of programmable site-specific nucleases has significantly facilitated targeted genome editing in a wide range of organisms and cultured cell types (Joung and Sander, 2013; Ran et al., 2013; Urnov et al., 2010). These customized nucleases induce DNA double strand breaks (DSBs) at desired genomic loci, triggering the endogenous DNA repair machinery through two competing pathways: error-prone nonhomologous end-joining (NHEJ), leading to insertion/deletion mutations (Indels), or homology-directed repair (HDR), which can be coopted to introduce precise nucleotide alterations using a homologous DNA template (Jasin, 1996; Rouet et al., 1994). Among various customized nuclease systems developed so far, the transcription activator-like effector (TALE) nuclease (TALEN) and the clustered, regularly interspaced, short palindromic repeat (CRISPR) technologies have emerged as powerful and versatile tools for genome editing in hPSCs.

The DNA target specificity of TALENs is guided by the TALE DNA binding domain. Originally discovered in the plant pathogenic bacteria *Xanthomonas*, TALEs can bind to the promoter of various genes of the plant host, hijacking the transcriptional machinery to promote bacterial infection (Rossier et al., 1999; Szurek et al., 2001). The DNA-binding domain of TALE is composed of ~34 amino acids repeats (TALE repeats) arranged in tandem. Each repeat contains two variable adjacent amino acids called the “repeat variable diresidue” (RVD), which determine the single base-recognition specificity. Thus, each TALE repeat independently specifies one target base (Boch et al., 2009; Moscou and Bogdanove, 2009). To introduce DSBs, TALENs are designed as pairs, recognizing the genomic sequences flanking the target site. Each TALEN consists of a programmable, sequence-specific TALE DNA-binding domain fused to the cleavage domain of the bacterial endonuclease FokI. The binding of a TALEN pair to DNA allows FokI dimerization and DNA cleavage (Cermak et al., 2011; Miller et al., 2011).

Recently, the CRISPR technology has been developed for genome engineering in mammalian systems (Cho et al., 2013a; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013b; Wang et al., 2013). The CRISPR/Cas system is derived from *Streptococcus pyogenes* where it functions as part of an immune system to provide acquired resistance against invading viruses (van der Oost et al., 2014). CRISPR/Cas-mediated genome engineering requires two components: the constant RNA-guided DNA endonuclease Cas9 protein required for DNA cleavage, and a variable CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) duplex that specifies DNA target recognition (Jinek et al., 2012). Most applications now replace the crRNA/tracrRNA duplex with a single chimeric guide RNA (gRNA), which works more efficiently than the original duplex design (Hsu et al., 2013; Jinek et al., 2012).

gRNA directs Cas9 for DNA cleavage at the target genomic locus, a 20 nt sequence (protospacer) followed by an NGG motif (protospacer-associated motif or PAM, where N

can be A, T, G or C), and DNA cleavage occurs 3 bp upstream of the PAM sequence. In our experience with hPSCs, the CRISPR/Cas system tends to outperform TALENs, which has also been observed by others in hPSCs and other cell types (Ding et al., 2013). Compared to TALENs, the CRISPR/Cas system is easier to engineer and simplifies multiplexing. However, there have also been concerns regarding its off-target effects (Cho et al., 2013b; Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013), which will be discussed further in Section 6.

A number of studies have now used CRISPR/Cas to establish modified clonal lines with variable efficiencies. Several studies use HDR-mediated editing to target a selectable marker into the locus of interest, which allows enrichment of correctly targeted cells after selection (An et al., 2014; Hou et al., 2013; Ye et al., 2014). Although efficient, the construction of the targeting construct could be time consuming, and it is often desirable to remove the selectable marker to allow more precise modeling of the disease conditions. Alternatively, the CRISPR/Cas system also supports efficient NHEJ or HDR-mediated genome editing without the need for drug selection (Ding et al., 2013; Gonzalez et al., 2014; Horii et al., 2013; Wang et al., 2014).

To further improve the efficiency, and to also achieve multiplexable and inducible genome editing in hPSCs, we have developed a genome-engineering platform called iCRISPR (Gonzalez et al., 2014). Through TALEN-mediated gene targeting, hPSC lines are engineered for doxycycline-inducible expression of Cas9 (referred to as iCas9 hPSCs). Upon doxycycline treatment, these lines can then be transfected with: a) a single or multiple gRNA(s) to generate biallelic knockout hPSC lines for individual or multiple genes; b) a gRNA together with a HDR template to generate homozygous knockin alleles; c) a gRNA at specific stages of hPSC differentiation to achieve inducible gene knockout.

Below we describe an optimized protocol for the establishment of the iCRISPR platform through TALEN-mediated targeting of inducible Cas9 expression cassettes into the *AAVS1* locus of hPSCs (Fig. 1). We have successfully used this protocol on four different hPSC lines and obtained similar results: ~ 50% of the lines are correctly targeted with no additional random integrations. Next, we provide detailed protocols for using iCRISPR to achieve one-step knockout of one or multiple gene(s), “scarless” introduction of precise nucleotide alterations, as well as inducible knockout during hPSC differentiation. We also provide guidelines for the selection of CRISPR targeting sequence, and for the design of single-stranded DNA (ssDNA) HDR templates for introduction of specific nucleotide modifications. Based on our successful experience using both hESCs (including HUES8, HUES9 and MEL-1) and hiPSCs, we believe the methods described here are generally applicable to most hPSC lines with minor adjustment.

iCRISPR supports a wide range of genome-engineering applications, and once established, our optimized workflow enables the generation of clonal lines with desired genetic modifications in as little as one month. Genome editing in hPSCs may finally become a routine laboratory procedure instead of a difficult and time-consuming task.

2. Generation of iCas9 hPSCs

Compared with transient, plasmid-mediated expression, targeted integration and inducible expression of Cas9 from a “safe harbor” locus provides a precise and reliable approach to express the invariable component of the CRISPR/Cas system. In this configuration, Cas9-expressing hPSCs can be easily transfected with gRNAs due to their small size (~100 nucleotides), leading to reproducible and highly efficient genome editing in target loci.

We generate iCas9 hPSCs by TALEN-mediated gene targeting into the *AAVS1* (also known as *PPP1R12C*) locus (Fig.2A). This site has been shown to support robust and sustained transgene expression similar to the *Rosa26* locus in mice (Smith et al., 2008). A pair of TALENs (AAVS1-TALEN-L and AAVS1-TALEN-R) is designed to generate a DSB in the first intron of *PPP1R12C* in the human *AAVS1* locus (Hockemeyer et al., 2011) (Fig.2B). Then two donor template plasmids are co-electroporated with the *AAVS1* TALEN constructs. The Puro-Cas9 donor plasmid contains a doxycycline-inducible Cas9 expression cassette selectable with Puromycin, and the Neo-M2rtTA donor carries a constitutive reverse tetracycline transactivator (M2rtTA) expression cassette selectable with G418 (Geneticin) (DeKolver et al., 2010) (Fig.2C). Homology directed repair (HDR) of the DSBs allows simultaneous introduction of both Puro-Cas9 and Neo-M2rtTA cassettes into both *AAVS1* alleles *in trans*. Cas9 expression is then induced through doxycycline treatment in established clonal iCas9 lines (Fig. 2A).

2. 1. Vector design

2. 1. 1. TALEN vectors—Two TALEN vectors are designed to target the *AAVS1* locus based on gene targeting studies by Jaenisch and colleagues (Hockemeyer et al., 2011): the *AAVS1*-TALEN-L vector is designed to target CCCCTCCACCCCACAGT, and the *AAVS1*-TALEN-R vector is designed to target TTTCTGTCACCAATCCT (Fig.2B). These TALEN vectors can be generated following a PCR-based protocol (Sanjana et al., 2012). First, build a library of TALE monomers with complementary overhangs by PCR using vector templates from Addgene (32180, 32181, 32182 or 32183). Next, join monomers into hexamers corresponding to the target DNA sequence. Finally, link the hexamers together and clone into the full-length TALEN expression backbone (Addgene 32190). Amplify both TALEN vectors in recombination deficient bacteria strain Stbl3 (Life Technologies, C7373-03) to avoid potential recombination problems due to highly repetitive sequences in the plasmid.

2. 1. 2. Donor vectors—The elements constituting the Puro-Cas9 and the Neo-M2rtTA donor vectors are described in Fig.2C. The gene-trap SA-P2A-Puro or Neo cassettes are in frame with the first exon of *PPP1R12C*. In correctly targeted cells, puromycin or neomycin will be expressed from the endogenous promoter, thus reduces the background of random integrations upon selection. Amplify both vectors in Stbl3. In particular, amplify Neo-M2rtTA at a lower temperature (30°C) since it is prone to recombination.

2. 2. hPSC electroporation

The electroporation protocol is optimized for hPSCs cultured on feeders. We routinely culture hPSCs on irradiated mouse embryonic fibroblasts (iMEFs) in DMEM/F12 medium (Life Technologies, 11320-082) supplemented with 20% KnockOut Serum Replacement (Life Technologies, 10828-028), 1X Non-Essential Amino Acids (Life Technologies, 11140076), 1X GlutaMAX (Life Technologies, 35050079), 100 U/mL Penicillin/100 µg/mL Streptomycin (Life Technologies, 15070063), 0.055 mM 2-mercaptoethanol (Life Technologies, 21985023) and 10 ng/mL recombinant human basic FGF (Life Technologies, PHG0263). Cultures are generally passaged at 1:6 to 1:12 split ratios every 4-6 days using TrypLE Select enzyme (Life Technologies, 12563-011). Add 5 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (R&D, 1254) into the culture medium when passaging or thawing cells. It may be necessary to adjust the following protocol for hPSCs cultured under different conditions such as the feeder-free TeSR and Essential 8 culture conditions (Chen et al., 2011b; Ludwig et al., 2006).

1. **Day -1.** On the day before electroporation, change to hPSC medium with 5µM Rock inhibitor. Typically, there are $\sim 1 - 2 \times 10^7$ hPSCs in one 10 cm dish when it is confluent on Day 0, which is sufficient for one targeting experiment (need $\sim 1 \times 10^7$ hPSCs).

Seed irradiated DR4-MEF (ATCC, SCRC-1045) (DR4-iMEFs) on gelatinized 10 cm culture dishes (2×10^6 cells/10 cm dish). The number of dishes needed depends on the survival rate after electroporation, which is line dependent. We generally prepare three 10 cm dishes. DR4-MEFs are resistant to neomycin, hygromycin, puromycin, and 6-thioguanine thus can be used as feeders to support the growth of hPSCs with multiple drug selections.
2. **Day 0.** On the day of electroporation, dissociate hPSCs using TrypLE Select enzyme. Stop the reaction by adding double amount of hPSC medium. Pipette gently to break cell aggregates into single-cell suspension and then pass cells through a 40 µm cell strainer to filter out cell clumps.
3. Pellet cells at $200g \times 5$ min in room temperature (RT).
4. Gently resuspend cells in cold (4°C) PBS and adjust cell density to 12.5×10^6 cells/ml.
5. Add the following plasmid mix into 800 µl cell suspension and mix well but gently.

Plasmid	Amount
AAVS1-TALEN-L	5 µg
AAVS1-TALEN-R	5 µg
Puro-Cas9	40 µg
Neo-M2rtTA	40 µg
hPSC (12.5×10^6 cells/ml)	800 µl

We recommend including a negative control (without donor vectors) when performing these experiments for the first time.

6. Transfer the DNA/cell mixture into a 0.4 cm electroporation cuvette and keep on ice for 5 min.
7. Electroporate cells using Gene Pulser Xcell Electroporation System (Bio-Rad, 165-2660) at 250 V and 500 μ F (Costa et al., 2007). The time constant observed after electroporation is typically $\sim$ 9-13.
8. After electroporation, transfer cells into 15 ml conical tube with 5 ml pre-warmed hPSC medium. Handle cells gently when transfer, resuspend and plate cells after electroporation.
9. Pellet cells at $200g \times 5$ min RT. Make sure to remove all floating dead cells and debris, which may impair the viability of the surviving hPSCs.
10. Resuspend cells in 10 ml hPSC medium with Rock inhibitor gently and plate 5, 2.5 and 1×10^6 cells onto each of the three 10 cm dishes pre-seeded with DR4-iMEF. This ensures that at least one of the plates would have sufficient colonies at clonal density for colony picking.
11. **Day 1.** Change hPSC medium. It is normal to observe significant cell death.

2. 3. Selection and expansion of clonal lines

1. **Day 2 - 5.** Start G418 (Life Technologies, 10131-035) selection. Change media daily with freshly made G418 (50 μ g/ml) selection media for 4 days. We expect to observe $\sim$ 200 colonies at this point, whereas no colonies should be present in the negative control dish. The optimal concentration and duration of G418 treatment may need to be adjusted depending on the G418 batch and the hPSC line.
2. **Day 6.** 4 days after G418 selection, change to hPSC medium without G418. Supplement with additional DR4-iMEF (1×10^6 cells/10 cm dish), as iMEFs typically support hPSC growth for up to 7 days. Rock the plates gently to disperse the feeder cells.
3. **Day 7 - 9.** Start puromycin (Sigma, P8833) selection and change media daily with freshly made puromycin (0.5 μ g/ml) selection media for 3 days. One may need to adjust the concentration and duration of puromycin treatment depending on the puromycin batch and the hPSC line.
4. **Day 10 - .** Change to regular hPSC medium without puromycin from day 10 onward. hPSC colonies can be picked around day 11 - day 14 when they reach $\sim$ 2 mm in diameter. We typically observe $\sim$ 50 colonies in the 10cm dish with 2.5×10^6 hPSCs plated on Day 0.
5. Because AAVS1 targeting using this approach is highly efficient, we typically pick only 12-24 colonies from each targeting experiment. Using a stereomicroscope and a 23G needle (200 μ l pipet tip is also fine), mechanically disaggregate hPSC colonies into small pieces ($\sim$ 10 pieces per colony) and transfer cells directly into a 24-well plate pre-seeded with iMEFs. Pipet gently in the 24-well plate to further break hPSC colonies. Generally, it takes $\sim$ 30 min to pick 24 colonies.

6. Change medium daily until cells become confluent. Passage cells in each well of 24-well plates into two wells in 6-well plates.
7. When cells become confluent in 6-well plates, use one well for a frozen stock and the other well for genomic DNA extraction.

Freezing medium: 10% DMSO, 40% FBS and 50% hPSC medium.

2. 4. Genotyping by Southern blot

From our experience targeting four hPSC lines, the efficiencies of biallelic integration of the Puro-Cas9 and Neo-M2rtTA transgenes into the *AAVS1* locus are usually close to 100%. It is therefore unnecessary to perform prescreening using PCR. We recommend amplifying the clones directly to obtain enough DNA for Southern blot, which would not only identify clones with correct biallelic transgene integrations in the *AAVS1* locus but also discriminate clones carrying random integrations (representing approximately 50% of the clones).

1. *Digoxigenin (DIG) labeled Probe Synthesis.* We routinely perform Southern blot using nonradioactive probes. Two DIG-labeled probes are used for *AAVS1* Southern blot genotyping. A 3' external probe and a 5' internal probe (Fig. 3, EXT and INT). They are synthesized using the PCR DIG Probe Synthesis Kit from Roche (Roche, 11636090910).

For 3' external probe, PCR amplify the probe template from human genomic DNA with high-fidelity Herculanase II Fusion DNA Polymerase (Agilent, 600679) using 3'F and 3'R primers. Clone the PCR product using Zero Blunt TOPO PCR Cloning Kit (Life Technologies, 450245) and use a sequence verified clone as template for amplifying the DIG-labeled probe.

For 5' external probe, directly amplify the DIG-labeled probe using Puro-Cas9 donor as the template using 5'F and 5'R primers.

Primer	Sequence
3'F	ACAGGTACCATGTGGGGTTC
3'R	CTTGCTCACCTGGCGATAT
5'F	AGGTCCGTCTCCTCCACT
5'R	GTCCAGGCAAAGAAAGCAAG

PCR DIG-labeled probe synthesis mix

	Full-Labeled	Half-labeled	Un-labeled
ddH ₂ O	33.25	33.25	33.25
10X PCR buffer	5	5	5
PCR DIG synthesis Mix	5	2.5	0
dNTP stock	0	2.5	5
Primer Mix (10 µM)	5	5	5
Enzyme mix	0.75	0.75	0.75
Plasmid DNA (50pg/µl)	1	1	1
Total (µl)	50	50	50

PCR DIG-labeled probe cycling condition

Cycle number	Denature	Anneal	Extend
1	95 °C , 2 min		
2-31	96 °C, 30 s	60 °C, 30s	72 °C, 1 min
32			72 °C, 7 min

Examine the PCR product on 2% Agarose gel. **NOTE:** DIG-labeled DNA probe migrates slower than non-labeled DNA.

2. Extract genomic DNA from expanded iCas9 lines as well as non-targeted wild-type hPSCs using DNeasy Blood & Tissue Kit (Qiagen, 69504). Typically, confluent hPSCs in one well of a 6-well plate yield 25 - 50 µg genomic DNA.
3. Digest 10 µg genomic DNA in a 20 µl reaction at 37°C over night using 20U of BgIII (for 3' external probe hybridization) or 20U of SphI (for 5' internal probe hybridization) (Fig. 3).
4. Run digestion samples on a 1% TAE agarose gel (including a lane with a DNA ladder). Migrate at 80V for 8 h. Image the gel in parallel with a ruler for scaling the image and determining band size upon film development.
5. Incubate the gel in denaturation buffer (1.5 M NaCl and 0.5 M NaOH) for 30 min at RT to denature DNA. If your lading buffer contains bromophenol blue, it should turn green during this step.
6. Remove denaturation buffer, wash for 1 min with ddH₂O before incubating the gel in neutralization buffer (1.5 M NaCl, 0.5M Tris and 0.001 M EDTA, pH=6.9) for 30 min at RT, and incubate for another 15 min with fresh neutralization buffer.
7. *Transfer.*
 1. Pre-incubate the Hybond-N membrane (GE healthcare, RPN203N) in 10x SSC buffer for 5 min.
 2. Mount the gel as described before for capillarity transfer (Maniatis et al., 1982). Make sure there is no bubble between the gel and membrane.
 3. Transfer overnight with 10x SSC buffer.
8. After transfer, wash the membrane in NaPi buffer (1M Na₂HPO₄, pH=7.2) for 5 min.
9. Fix DNA on the membrane by baking at 80 °C for 2 h.
10. Place the membrane in a hybridization tube (Fisher, K736500-3515). Add 20 ml NaPi buffer. Incubate for 5 min in rotating oven at RT.
11. Pre-hybridize the membrane with 20 ml hybridization buffer 1 h in a rotating oven at 65°C.

Hybridization buffer (500 ml)

Component	Amount
NaPi 1M pH 7.2	250 ml
SDS 20%	175 ml

EDTA 0.5M pH8	1 ml
ddH ₂ O	74 ml

- 12. Probe preparation.** Denature 5 µl of the probe from Step 1 in 100 µl hybridization buffer in boiling water for 10 min. Then dilute the 100 µl probe in 20 ml hybridization buffer (pre-warmed at 65°C).

Note: After use, the probe can be collected and stored at -20°C and reused for at least 5 times. For second time use, thaw the diluted probe and denature in boiling water for 10 min.

- 13.** Remove pre-hybridization buffer and add 20 ml of the diluted probe to the hybridization tube. Hybridize the membrane in rotating oven at 65°C overnight.

- 14.** Wash the membrane twice with washing buffer at 65°C for 5 min.

Washing buffer (2000 ml)

Component	Amount
NaPi 1M pH 7.2	80 ml
SDS 20%	100 ml
ddH ₂ O	1920 ml

- 15.** Wash the membrane in DIG buffer I with 0.3% Tween at RT under gentle agitation for 5 min.

- 16.** Block the membrane in 20 ml DIG buffer I with 1% blocking reagent (Roche, 11096176001) at RT for 30 min in a sealed plastic bag.

- 17.** Centrifuge the anti-DIG-AP antibody (Roche, 11093274910) at 4°C for 5 min before use. Dilute 1 µl of the antibody in 20 ml DIG buffer I with 1% blocking reagent.

- 18.** Incubate the membrane with 20 ml of the diluted antibody at RT° for 30 min in a sealed plastic bag.

- 19.** Wash the membrane twice in DIG buffer I 0.3% Tween in a plastic container for 30 min.

- 20.** Wash the membrane with DIG buffer III (0.1 M NaCl, 0.1 M Tris, pH 9.5) for 5 min.

- 21. Chemiluminescent detection.** Incubate the membrane with 12 µl CDP-star (Roche, 11759051001) diluted in 2 ml DIG buffer III for 5 min.

- 22.** Remove excess liquid without drying. Transfer the membrane to a clean sealed bag. Expose with Amersham Hyperfilm (Fisher, 45-001-507) in Amersham Hypercassette (Fisher, 45-000-758). Exposure time varies with probe concentration and quality but is typically 30 min.

2. 5. Validation

Correctly targeted iCas9 lines are further validated through qRT-PCR for inducible expression of Cas9, immunohistochemistry to assess proper expression of the pluripotency

markers (e.g. OCT4, SOX2 and NANOG) and teratoma assay for functional assessment of pluripotency. We also recommend verifying that the newly established iCas9 cells have normal karyotypes.

2.5.1. RT-PCR Analysis

1. Treat iCas9 cells with or without doxycycline (2 µg/ml) (Fisher, BP26535) for 2 days.
2. Isolate total RNA using RNeasy Plus Mini Kit (Qiagen, 74134).
3. Synthesize cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, 4368814).
4. Perform quantitative PCR using SYBR Green low ROX Mix (Fisher, AB4322B) and 7500 Real Time PCR System (Life Technologies, 4351104). The following primers are used. GAPDH is used as the internal control. Doxycycline treatment typically induces ~ 1,000 fold increase in Cas9 mRNA level.

Primer	Sequence
Cas9-F	CCGAAGAGGTCGTGAAGAAG
Cas9-R	GCCTTATCCAGTTCGCTCAG
GAPDH-F	GGAGCCAAACGGGTCATCATCTC
GAPDH-R	GAGGGGCCATCCACAGTCTTCT

2.5.2. Immunohistochemical analysis of pluripotency marker expression

1. Rinse cells with PBS once and fix cells with 4% PFA (Fisher, 50980495) in PBS for 10 min directly in the culture plate.
2. Permeabilize and wash cells three times with PBST (0.1% Triton X-100 in PBS) for 5 min.
3. Block cells with blocking buffer (5% serum* in PBST) for 5 min.
* Serum should be from species different from primary antibody.
4. Incubate with primary antibody diluted in blocking solution at RT° for 1 hour (or at 4 °C overnight).

Antibody	Company	Dilution ratio
Goat anti-OCT4	Santa Cruz, sc-8628	1:100
Rabbit anti-NANOG	Cosmobio Japan, REC- RCAB0004P-F	1:100
Goat anti-SOX2	Santa Cruz, sc-17320	1:100

5. Remove primary antibody and wash three times with PBST for 5 min.
6. Incubate with fluorescence conjugated secondary antibody diluted in blocking solution at RT for 1 h (also add DAPI if nuclear staining is desired).
7. Remove secondary antibody and wash cells three times with PBST for 5 min.

2.5.2. Teratoma Assay

1. Expand iCas9 lines to 100% confluency on 10 cm dishes.
2. Add 7 ml collagenase type 4 (Life Technologies, 17104-019) working solution (1mg/ml in DMEM) and incubate at 37°C for 10 min.
3. Aspirate collagenase solution.
4. Add 5 ml hPSC medium.
5. Scrape cells with cell scraper (Fisher, 087711A) and collect cell suspension into 15 ml conical tube.
6. Pellet cells at 200g for 5 min.
7. Resuspend cell pellet in 400 µl PBS.
8. Inject subcutaneously 100 µl cell suspension into the right hind leg of an anesthetized immunocompromised mice. We routinely use severe combined immunodeficient (SCID) mice for this purpose.

3. Generation of knockout hPSCs using iCRISPR

3. 1. gRNA design

NHEJ-mediated repair of DNA DSBs leads to random Indel mutations, which is useful for generating loss-of-function mutations, or knockouts. Thus the design of the gene targeting strategy aims at generating premature stop codons through the creation of frameshift Indel mutations, and the target sequence need to be strategically chosen to maximize the possibility of disrupting the function of the corresponding protein. It is important to identify all possible splice variants of the gene of interest, and design gRNAs to target a functional region that is at least present in the isoform relevant for the study. For well-annotated genes, we recommend targeting sequences upstream of an essential functional domain of the corresponding protein. Alternatively, one may target a region downstream of the start codon of the major isoform. The second strategy may sometimes create a hypomorphic allele instead of a null allele if an alternative start site is used to produce a partially active protein.

Proceed to designing specific gRNAs to target the genomic region of interest after a targeting strategy is decided. We routinely use the CRISPR design tool developed by the Feng Zhang group at MIT (<http://crispr.mit.edu/>). The software not only identifies all possible CRISPR targets in an input DNA sequence but also uncovers potential off-target sites, thus predicting gRNAs with the highest targeting specificity. For each gene of interest, we routinely design 3 gRNAs and in most cases all of them display efficient cutting (> 20% by T7EI assay) in target loci.

3. 2. gRNA production

3. 2. 1. PCR amplification of *in vitro* transcription (IVT) DNA templates—

Different from most commonly used approaches that start with the construction of a gRNA plasmid, we have found that it is faster and more cost-effective using an oligo template. This method also allows easy scaling up for higher throughput knockout studies. Design a 120

nucleotide (nt) oligo including the T7 promoter sequence (blue and underlined) and the variable 20 nt crRNA recognition sequence (N)₂₀ followed by a constant chimeric guide RNA sequence (Fig. 4A). PCR amplify the oligo using T7 F and Tracr R universal primers.

Primer	Sequence
T7 F	TAATACGACTCACTATAGGG
Tracr R	AAAAGCACCGACTCGGTGCC

PCR Reaction mix (50 µl)

Component	Amount
ddH ₂ O	35.5 µl
5X Herculase II reaction buffer	10 µl
dNTP mix (25 mM)	0.5 µl
T7 F (10 µM)	1.25 µl
Tracr R (10 µM)	1.25 µl
T7-gRNA IVT template (25 nM)	1 µl
Herculase II fusion DNA polymerase	0.5 µl

PCR cycling conditions

Cycle number	Denature	Anneal	Extend
1	94 °C , 2 min		
2-36	94 °C, 20 sec	N* °C, 20 sec	72 °C, 30 sec
37			72 °C, 2 min

* Annealing temperature of the T7EI primer pair, ideally between 55°C and 65°C

3. 2. 2. *In vitro* transcription and purification of gRNAs—Use the MEGAshortscript T7 Transcription kit (Life Technologies, AM1354) to generate gRNAs.

In vitro transcription mix (20 µl)

Component	Amount
T7 ATP	2 µl
T7 CTP	2 µl
T7 GTP	2 µl
T7 UTP	2 µl
T7 10X buffer	2 µl
T7 enzyme mix	2 µl
PCR amplified T7-gRNA IVT template	8 µl

Incubate for 4 hours at 37°C

Add 1ul TURBO DNase and incubate 15 minutes at 37°C

Proceed to RNA purification using the MEGAclean Transcription Clean-Up Kit (Life Technologies, AM1908) following manufacturer's instructions, and elute gRNAs (typically ~ 50 - 100 µg) in 100 µl RNase-free water. When possible adjust concentration to 320 ng/µl (10 µM) and store at -80°C until use.

3. 3. Single or multiplex gRNA transfection in hPSCs

We routinely transfect hPSCs cultured in 24-well dishes in duplicating wells: one well is used for T7EI and/or RFLP assays to confirm efficient mutagenesis, and the other well is used for replating and colony picking for establishment of clonal knockout lines. We also recommend using three different cell densities for transfection to achieve an optimal balance between Indel efficiency and cell survival.

Day 0. Plate iMEFs on a gelatin-coated 24-well plate and treat 60% confluent iCas9 hPSC with hPSC medium containing 2 µg/ml doxycycline.

Day 1. Dissociate hPSCs using TrypLE and resuspend at 0.2, 0.4 and 1×10^6 cells/ml in hPSC media with 5 µM ROCK inhibitor and 2 µg/ml doxycycline. Replate on a 24-well plate: 2×0.5 ml dilution per well. For each dilution, plate an additional well to serve as non-transfected control for T7EI and RFLP assays.

1sttransfection. For each 0.5 mL cell suspension, prepare separately:

Mix 1: 25 µl Opti-MEM + 0.5 µl gRNA (160 ng)

Mix 2: 25 µl Opti-MEM + 1.5 µl Lipofectamine RNAiMAX (Life Technologies, 13778-150)

Mix 1+2, incubate 5 min at RT and add dropwise into dissociated hPSCs.

The final concentration of the gRNA is 10 nM.

Day 2. Change hPSC medium with 2 µg/ml doxycycline.

2ndtransfection (optional). Same conditions as 1st transfection.

Day 3-4. Change media: 0.5 ml/well of hPSC medium.

For multiplexed gRNA transfection, use the same strategy but mix and transfect equal amounts of each gRNA keeping the sum equal to 160 ng/well.

3. 4. Assessment of Indel frequency

Two or three days after last gRNA transfection, extract genomic DNA from transfected and non-transfected control cells using DNeasy Blood & Tissue Kit. Adjust final concentration to 50 ng/µl.

3. 4. 1. PCR amplification of the CRISPR target region—PCR amplify a stretch of genomic region (~400–600 bp) flanking the CRISPR target site using the high-fidelity Herculanase II Fusion DNA Polymerase to avoid potential PCR induced mutations.

PCR Reaction mix (20 µl)

Component	Amount
ddH ₂ O	13.6 µl
5X Herculanase II reaction buffer	4 µl
dNTP mix(25 mM)	0.2 µl

Primer-F (10 μ M)	0.5 μ l
Primer-R (10 μ M)	0.5 μ l
genomic DNA (50 ng/ μ l)	1 μ l
Herculase II fusion DNA polymerase	0.2 μ l

PCR cycling conditions

Cycle number	Denature	Anneal	Extend
1	94 °C , 2 min		
2-31	94 °C, 20 sec	60 °C, 20 sec	72 °C, 1 min
32			72 °C, 2 min

3. 4. 2. Quantification of Indels through T7EI assay—For this step we use the T7 Endonuclease I (New England Biolabs, M0302S)

3. 4. 2. 1. Hybridization

Mix (16 μ l)	
Component	Amount
PCR product (do not purify)	8 μ l
NEB Buffer 2 10X	1.6 μ l
ddH ₂ O	6.4 μ l

Conditions (use thermocycler)

95°C, 5 min, 95–85°C at –2°C/s, 85–25°C at –0.1°C/s Hold at 4°C

3. 4. 2. 2. Digestion

Mix (20 μ l)	
Component	Amount
Hybridized PCR product	16 μ l
NEB Buffer 2 10X	0.4 μ l
T7EI (10 U/ μ l)	0.2 μ l
ddH ₂ O	3.4 μ l

Incubate at 37°C for 30 min, immediately proceed to Indel quantification or store at -20°C to inhibit the reaction.

3. 4. 2. 3. Quantification: Resolve samples by electrophoresis through a 2.5% ethidium bromide stained agarose gel. Use Xylene Orange loading dye to avoid overlap with the expected bands. Run the gel at 5 V/cm enough time to separate the digested bands. The background decreases with migration time and bands resolve better.

UV illuminate the gel and image (avoid saturation) with a Gel Doc gel imaging system (Bio-Rad). Indel quantification is calculated based on relative band intensities using ImageJ software (National Institutes of Health, Bethesda, MD) (Fig. 3B).

Indel percentage is determined by the formula: $100 \times (1 - (1 - (b + c) / (a + b + c))^{1/2})$, where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product (Hsu et al., 2013) (Fig. 3B).

3. 4. 3. Quantification of Indels through RFLP assay—One may also design CRISPRs so that the Cas9 cleavage site (3 bp 5' of the PAM sequence) is in close proximity (~5 bp) to a restriction site, thus a restriction fragment length polymorphism (RFLP) assay can be performed to quantify Indel frequency. The assay requires the same PCR amplification of the CRISPR cutting region (see Section 3. 4. 1). Since this assay directly measures the loss of a restriction site, it is more sensitive at detecting Indels compared to the T7EI assay as long as the restriction site is located within ~5 bp of the Cas9 cleavage site.

3. 4. 3. 1. Digestion: Incubate for at least 1h, and proceed RFLP quantification or store at -20°C.

3. 4. 3. 3. Quantification: Follow the same approach described in Section 3. 4. 2. 3.

Indel percentage is determined by the formula: $100 \times a / (a+b+c)$ (Fig. 3C).

3. 5. Clonal expansion of knockout lines

After performing T7EI and/or RFLP assay using the cells from one of the duplicating wells, identify the best transfection conditions (with high Indel efficiency and good cell survival) and use the corresponding well for clonal expansion of mutant lines. We generally proceed to clonal expansion when Indel frequencies >20% are observed, which is usually achieved in one out of three gRNAs tested.

3. 5. 1. Replating and Colony picking—2-3 days after the last gRNA transfection, hPSCs are dissociated into single cells and replated at ~2,000 cells per 10 cm dish. Cells are allowed to grow until colonies reach ~ 2 mm in diameter (~10 days).

Single colonies are then picked and plated in an uncoated 96-well plate containing 100 ul of hPSC medium with Rock inhibitor. Each clone is mechanically disaggregated by gently pipetting up and down 5 times and replated in duplicated 96-well plates (50 ul each) pre-seeded with iMEFs and containing 100 ul of hPSC medium with Rock inhibitor.

Alternatively, colonies could first be cultured in one 96-well plate and then split into two 96-well plates when it is confluent (in ~ 5 days).

Depending on the frequencies of Indel observed by T7EI and RFLP assays pick between 48 (> 20% targeting efficiency, applicable to making single-gene knockouts) to 288 clones (< 10% targeting efficiency, applicable to making triple-gene knockouts). Media change and passaging using multichannel aspirator and dispenser pipettes greatly facilitates this step.

3. 5. 2. Colony Screening—Grow duplicated 96-well plates with daily change of fresh hPSC media. Use with one plate for analysis in 3 days and the other plate for maintenance. One may use RFLP to identify clonal lines when feasible. We routinely screen for mutant clones through direct sequencing of PCR products using a simplified procedure. This

procedure involves first using a simple protocol (without phenol/chloroform extraction) to extract genomic DNA, then PCR amplifying the region of interest, and finally sequencing the unpurified PCR product. Since the entire procedure can be accomplished using multichannel pipettes, it enables the screening of a large number of clones rapidly. In our experience, this procedure works well as long as the primers are pre-tested before the targeting experiment, and it is feasible for a single person to process up to 288 samples (three 96-well plates) at a time

3. 5. 2. 1. Lysis: Remove media from the wells, wash once with PBS and add 50 μ l of Lysis buffer.

Lysis buffer (50 μ l/well)

Component	Amount
Proteinase K (10 mg/ml)	1.5 μ l
JumpStart PCR buffer 10X (Sigma)	5 μ l
ddH ₂ O	43.5 μ l

To avoid excessive evaporation, seal the plate using a qPCR film sticker and incubate O/N at 55°C.

The next day, mix and transfer the cell lysates into a 96-well PCR plate and incubate for 10 min at 96°C in a thermocycler to inactivate Proteinase K.

3. 5. 2. 2. PCR and sequencing: PCR amplify using the same primers as for T7EI or RFLP assay and 1 μ l of cell lysate as template. Use 1 μ l of the PCR product for Sanger sequencing using a primer binding inside the PCR product. To generate knockout lines, amplifying clones with frameshift Indel mutations for frozen stocks. We also recommend amplifying and freezing a couple of wild-type clones from the same targeting experiment to serve as control lines for further experiments.

In the case of heterozygous and compound heterozygous mutants, the traces can be complicated to interpret due to overlapping peaks. To further characterize the mutations present in each allele, we amplify the gRNA target region of these clones with high-fidelity Herculanase II Fusion DNA Polymerase using T7EI PCR primers and clone the PCR product using Zero Blunt TOPO PCR Cloning Kit (Life Technologies, 450245). Sanger sequencing of 10 individual bacterial colonies allows mono-allelic characterization of the mutations.

If the sequencing turnaround time is relatively short (e.g. within a day), it is possible to directly amplify the desired mutant clones from the maintenance plate before they become confluent. Alternatively, if the sequencing turnaround time is longer, we recommend freezing down cells in the entire maintenance plate. Dissociate cells with 25 μ l TrypLE Select enzyme in each well of 96-well plates, stop the reaction by adding 50 μ l hPSC medium per well. Then add 75 μ l 2 $\times$ freezing medium directly into the well and mix gently. Wrap the plate with plastic film, put into a Styrofoam box and transfer to -80 °C. The frozen plate can be stored for at least 3 months at -80 °C.

3. 5. 3. Validation

3. 5. 3. 1. Validation of the mutant alleles: It is important to realize that CRISPR-mediated Indel mutations may create complete loss-of-function, partial loss-of-function, or occasionally gain-of-function (e.g., dominant-negative or neomorphic) alleles. Therefore, it is important to perform additional analysis to determine the exact nature of individual mutant alleles. For instance, Western blot may be performed to verify the absence of the wild-type protein in a mutant clone, as long as a good antibody is available for the protein of interest. One may also validate the absence of a functional protein based on its known biological function. For instance, based on the requirement of TET proteins for catalyzing the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Ito et al., 2010; Tahiliani et al., 2009), quantification of the 5hmC levels can be used to verify the TET1/2/3 triple-targeted hESC lines (Gonzalez et al., 2014).

3. 5. 3. 2. Off-target analysis: Due to mismatch tolerance of CRISPR gRNA pairing (Hsu et al., 2013), there are concerns about the off-target mutagenic effects of the CRISPR/Cas system. Recently, whole-genome sequencing of CRISPR targeted hPSC clonal lines has detected very rare off-target mutations attributable to CRISPR. Thus off-target mutations may not be a significant concern for disease modeling and other applications using hPSCs (Veres et al., 2014).

One may identify potential ectopic gRNAs targets using the CRISPR design tool (<http://crispr.mit.edu/>). The most likely off-targets falling in gene coding sequences (4-5 sites per gRNA-mediated targeting experiment) can be analyzed through sequencing using the same approach depicted to analyze mutations at gRNA target sites. PCR amplify a stretch of genomic region (~500 bp) flanking the CRISPR off-target site using Herculase II Fusion DNA Polymerase and analyze by Sanger sequencing using a primer binding inside the PCR product and allowing amplification of the gRNA off-target site.

4. Generation of precise genetic alterations using iCRISPR

Without a DNA repair template, NHEJ repair of DSBs introduces Indels into the genome. However, in the presence of a DNA repair template, homology-directed repair (HDR) of DSBs could lead to precise nucleotide alterations of the hPSC genome, which is important to either create disease-specific variants in wild-type cells or correct disease-associated mutations in patient cells. Compared with double-stranded DNAs (dsDNA), synthetic ssDNAs (~ 80 - 200 nt) are easy to produce and can be used as DNA repair template without selection (Chen et al., 2011a).

4. 1. Design of ssDNA as HDR templates

To introduce specific nucleotide alterations, we co-transfect gRNA with ssDNA as HDR template.

1. Design 2 - 3 gRNAs in close proximity to the genetic alteration locus.
2. Design ssDNA templates containing the genetic alternation flanked by ~ 40 - 80 nt of homology on each side of the space between the genetic alternation site and the CRISPR cutting site (3 nt 5' of PAM sequence) (Fig. 5A). Given the close

proximity of the gRNAs designed, only one ssDNA template is necessary in most cases.

3. We highly recommend introducing a silent mutation to eliminate the PAM sequence in the ssDNA template. This would prevent further editing of correctly modified alleles (Fig. 5B, G>A in the PAM sequence).
4. If the genetic alternation introduces or disrupts a restriction site, HR efficiency can be assessed through RFLP analysis (Fig. 5A, C>A introduces a BgIII site).
5. If the genetic alternation doesn't introduce or disrupt a restriction site (Fig. 5C, A>G) we also recommend introducing a silent mutation that creates a new restriction site (Fig. 5C, T>C generates a SacII site) to enable assessment of HR efficiency through RFLP.

4. 2. ssDNA/gRNA cotransfection in hPSCs

1. **Day 0: Pretreatment with doxycycline.** Treat iCas9 cells with doxycycline for 24 h before transfection when hPSCs are ~ 60% confluent so that on the day of transfection, Cas9 will already be expressed at high level. Seed iMEFs on a gelatin-coated 24-well plate.
2. **Day 1: 1st transfection.** On the day of transfection, dissociate and replate cells as described in 3.3.

Perform co-transfection of gRNA and ssDNA in one well of a 24-well plate as follows

Component	Amount (μl)
Opti-MEM	50
RNAiMAX reagent	1.5
320 ng/μl gRNA	0.5
300 ng/μl ssDNA	2.5

3. **Day 2: 2nd transfection (optional).** 24 h after the 1st transfection, change hPSC medium with doxycycline and repeat the transfection as Day 1.
4. **Day 3-4.** Change hPSC medium.

4. 3. Establishment

HDR frequency will be assessed through RFLP assays as described in Section 3.4.3, followed by procedures to establish clonal lines as described in Section 3. 5.

5. Inducible gene knockout in hPSCs using iCRISPR

Inducible gene knockout during differentiation of hPSCs into specific cell types is of great importance for studying genes with pleiotropic effects. With iCRISPR platform, inducible gene knockout could be achieved by inducible Cas9 expression and temporally regulated delivery of gRNA due to the low toxicity of lipid-mediated gRNA transfection (Fig. 6A). Alternatively, inducible gene knockout could also be achieved through generation of iCr lines including a constitutive gRNA expression module in addition to the doxycycline-inducible Cas9 expression cassette (Puro-iCr) targeted into the *AAVS1* locus (Fig. 6B).

Compared with temporally regulated delivery of gRNA, generation of iCr lines allows inducible gene targeting in all the cells upon doxycycline treatment.

5. 1. Inducible gene knockout through gRNA transfection

1. Differentiate iCas9 into target cell types following established protocol.
2. FACS sort target cells when cell surface marker(s) is available and replat cells.
3. Treat cells with doxycycline 24 h before transfection.
4. Perform gRNA transfection as described in 3.3.

5. 2. Inducible gene knockout through using iCr hPSC lines

To generate iCr hPSC lines for inducible gene knockout, we use the same cloning approach developed by Cong et al. (Cong et al., 2013) to insert the CRISPR target sequence ($N_{(19)}$) into a single BbsI site found in the pEntr-Cr plasmid (Fig. 7A, B)

Then through LR reaction using pEntr-Cr and Puro-iDEST plasmids, the gRNA expression module and Cas9 expression cassette is transferred from pEntr-Cr to Puro-iDEST, generating the Puro-iCr donor (Fig. 7C).

1. *pEntr-Cr*. pEntr-Cr plasmid was constructed by introducing the chimeric gRNA expression module and Cas9 coding sequence, PCR amplified from pX330 (Cong et al., 2013) ,into a pEntr plasmid (Fig. 7A).
2. *Puro-iDEST*. Puro-iDEST plasmid was constructed by replacing the Cas9 coding sequence in the Puro-Cas9 donor with a gateway destination cassette (Life Technologies, Gateway system) (Fig. 7C).
3. *Puro-iCr*. donor CRISPR/Cas9 expression vectors targeting specific genomic loci are generated through LR reaction between *pEntr-Cr* and *Puro-iDEST* (Invitrogen, Gateway Technology) (Fig. 7C).

6. Conclusions and Future directions

Below we discuss expected results, common issues and considerations, and potential additional use and extension of the iCRISPR platform.

6. 1. Anticipated results

Using iCRISPR, we have successfully mutated 20 genes so far. Although the efficiencies vary depending on the genomic locus, in most single-gene targeting experiments, we found 20 to 60% of the clones with both alleles mutated (including in-frame and frameshift mutations). In cases where we performed multiplexed gene targeting, we obtained triple biallelic mutant clones with 5 - 10% efficiencies. We have also performed ssDNA-mediated HDR with several genes with the efficiencies of obtaining homozygous knockin clones ranging from 1 to 10% of the lines examined. To inactivate genes in a temporal or tissue-specific manner, we have observed up to ~75% mutation rate in doxycycline-induced iCr cells.

There is usually a good correlation between the T7EI/RFLP assays and the number of mutant lines recovered upon clonal expansion. This highlights the importance of performing these assays in parallel with clonal expansion. One may also test several gRNAs through performing T7EI/RFLP assays, and pick the most efficient one(s) for establishing mutant lines.

6. 2. In-frame mutations

Occasionally we have encountered situations where the majority of mutant alleles carry the same in-frame mutations. This appears to be caused by microhomology-mediated repair that utilizes short stretches of repeated sequences flanking the DSB site. This issue can be overcome simply by designing gRNAs that target different sequences. One may also attempt to predict the possibility of microhomology-associated Indels at CRISPR target sites, and potentially make use of microhomology-mediated DNA repair to maximize the frequency of frameshift mutations (Bae et al., 2014).

Of note, a failure to recover frameshift mutant clones may also reflect a requirement of the gene for the survival or self-renewal of hPSCs. One should consider this possibility when multiple gRNAs with high Indel efficiencies have produced only in-frame biallelic mutations, and there is no apparent association with microhomology-mediated DNA repair. In this situation one may perform inducible knockout (see Section 5) or knockdown by RNA interference.

6. 3. Cross contamination

While we have been able to routinely generate mutant lines that are homogeneous with respect to the mutated allele(s), it is occasionally possible that a given mutant line has arisen from more than one cell. Therefore, care should be taken to pick only well-spaced colonies for line establishment, and to avoid cross contamination when expanding multiple clonal lines. After a clonal line is established, we recommend confirming the presence of mutant allele(s) through TA cloning. One may further ensure the establishment of clonal lines through subcloning. Although not routinely used in our laboratory, single-cell deposition flow cytometry can be used to assist this procedure (Davis et al., 2008).

6. 4. Time and throughput considerations

Based on the generally high targeting efficiencies obtained with iCRISPR, the analysis of ~24 - 48 colonies should be sufficient to establish multiple mono- and biallelic mutant lines for each gene, and the entire process takes about 1 – 2 months. We have found that it is feasible for a trained individual to pick around 288 colonies at a time, which takes approximately 3 hours. With this throughput, an experienced individual could also mutant several individual genes in parallel.

6. 5. Off-target considerations

iCRISPR offers an efficient platform to generate isogenic wild-type and mutant hPSC clones allowing rapid functional studies of single and multiple genes. However, studies performed in pools of immortalized cell lines suggest that both TALEN and CRISPR/Cas systems can induce unwanted mutations at loci sharing high sequence similarity with the target site (Cho

et al., 2013b; Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013). These studies raise concerns that off-target effects could make it difficult to interpret findings from studies using TALEN or CRISPR/Cas systems. Recent studies have identified only low incidence of off-target mutations from whole-genome sequencing analysis in individual hPSC clones obtained through genome manipulation with TALENs or CRISPR/Cas (Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014). Therefore, although off-target mutations are still a risk for therapeutic applications, they are not a significant concern for biological studies and disease modeling.

Nevertheless, unintended off-target mutations could still obscure functional studies in hPSCs. To minimize confounding phenotypes introduced by off-target effects, we recommend generating independent mutant lines using at least two different gRNAs targeting different sequences in the same gene. Wild-type clones identified from the same screen of gRNA transfected hPSCs will be used as control lines. One should also consider complementing the loss-of-function approaches with rescue experiments when feasible. To further demonstrate the generality of any experimental finding, it may be desirable to generate mutant lines using more than one hPSC iCas9 line.

6. 6. Additional use and extension of the iCRISPR platform

The iCRISPR platform is likely to facilitate other types of genome editing in hPSCs. Also, by replacing Cas9 with other Cas9 variants such as dCas9, dCas9-KRAB or dCas9-VP16 (Gilbert et al., 2013; Qi et al., 2013), one may repurpose iCRISPR for gene regulation. Among the many possible applications of CRISPR/Cas, we would like to highlight two areas that we feel have not yet been widely discussed. First, CRISPR/Cas may be used to create deletions in non-coding RNAs or gene regulatory regions such as promoters and enhancers. To efficiently generate regulatory mutants using iCRISPR, one may design gRNAs to disrupt the binding site (usually a short sequence) of a DNA-binding protein including but not restricted to the basal transcription machinery or a tissue-specific transcription factor. Alternatively, specific protein binding sites may be disrupted or modified through ssDNA-mediated HDR.

iCRISPR may also facilitate the generation of more complex genomic modifications, such as the creation of reporter alleles through HDR-mediated gene targeting using long donor DNA templates encoding protein tags or fluorescent proteins. Conventional approach to generating knockin reports requires a drug-resistance cassette due to the low targeting efficiency. However, an additional step is often necessary to remove the drug-resistance cassette, the presence of which often affects reporter gene expression. Although similar targeting experiments have been performed with CRISPR/Cas zygote injection in mice and rats (Ma et al., 2014; Yang et al., 2013), this has not yet been achieved in mouse or human pluripotent stem cells. With sufficiently high targeting efficiencies, we imagine it might be possible to perform this type of targeting in hPSCs without drug selection, which would be a significant technical advance in our opinion.

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The iCRISPR platform for rapid genome editing in hPSCs

Figure 1. The iCRISPR platform for rapid genome editing in hPSCs

Instead of transient expression of Cas9 and gRNAs from electroporated plasmids in hPSCs, targeted integration and inducible expression of Cas9 into the *AAVS1* locus provides a precise and reliable approach to express the invariable component of the CRISPR/Cas system (Generation of iCas9 hPSCs, Section 2). Then, gRNAs targeting specific loci are designed and synthesized by PCR amplification of *in vitro* transcription DNA templates (gRNA production, Section 3.1 and 3.2). Due to their small size (~100 nucleotides), transfection of iCas9 hPSCs with gRNAs is highly efficient, thus could lead to reproducible and highly efficient gene knockout in hPSCs (Generation of hPSC knockouts by gRNA transfection, Section 3.3).]

Figure 2. Generation of iCas9 hPSC through TALEN-mediated gene targeting into the AAVS1 locus

(A) TALEN-mediated gene targeting of Puro-Cas9 and Neo-M2rtTA into the AAVS1 locus. Homology directed repair (HDR) of the DSBs introduced by a pair of TALENs allows simultaneous introduction of both Puro-Cas9 and Neo-M2rtTA cassettes into both AAVS1 alleles *in trans*. Cas9 expression is then induced through doxycycline treatment in established clonal iCas9 lines. (B) The TALEN-L vector is designed to target CCCCTCCACCCACAGT, and the TALEN-R vector is designed to target TTTCTGTCACCAATCCT. Dimerization of the FokI domain induces DSB in the spacer between the TALEN binding sites. (C) Vector maps of Puro-Cas9 and Neo-M2rtTA vectors. HA-L and HA-R: Left and right homology arm; SA: splicing acceptor; 2A: self-cleaving 2A peptide; pA: polyadenylation signal sequence; attB1 & attB2: GATEWAY attB sequence; TRE: Tetracycline Response Element; Amp^r: Ampicillin resistance gene; Co1EI: replication origin.]

Southern blot genotyping of AAVS1-targeted hPSC iCas9 lines

Figure 3. Southern blot genotyping of AAVS1-targeted hPSC iCas9 lines
DNA is digested with either BglIII (B), for 3' external probe hybridization, or SphI (S), for 5' internal probe hybridization. BglIII digestion and 3' external probe hybridization yield bands of 12406, 7409 and 4984 bp for wild-type, Neo-M2rtTA, Puro-iCas9 (or Puro-iCr); whereas SphI digestion and 5' internal probe hybridization yields bands of 6492, 3492 and 3781 bp respectively.]

Figure 4. gRNA production, T7EI and RFLP assay

(A) The 120 nt ssDNA gRNA production template. T7: T7 promoter sequence; N(20): 20 nucleotide gRNA complementary target sequence; (B) T7EI assay. Ctrl: None-transfection control. (C) RFLP assay.]

Design of homology-directed repair (HDR) ssDNA templates

Figure 5. Design of homology-directed repair (HDR) ssDNA templates

(A) gRNAs are designed close to the genetic alteration locus. Arrow ahead points to the Cas9 cutting site 3 nt 5' of PAM sequence. The ssDNA HDR ssDNA template contains the required genetic alteration (C>A, labeled in red) flanked by ~40 - 80 nt of homology on each side of the space between the genetic alteration site and the CRISPR cutting site. A new restriction site (BglII) introduced by the genetic alteration allows assessment of HR efficiency through RFLP analysis. (B) Introducing a silent mutation (G>A, labeled in green) in the PAM sequence would prevent further editing of correctly modified alleles. (C) When required genetic alteration doesn't generate a restriction site, introducing a silent mutation (T>C, labeled in green) nearby that creates a new restriction site (SacII) enables assessment of HR efficiency through RFLP.]

Figure 6. Inducible gene knockout in hPSCs using iCRISPR

(A) iCas9 hPSCs are first differentiated into required cell types. Induced expression of Cas9 with doxycycline treatment and gRNA transfection in differentiated cells result in inducible gene knockout. (B) In iCr hPSCs, a constitutive gRNA expression module is inserted 3' of the Cas9 expression cassette. Treat differentiated iCr hPSCs with doxycycline induces Cas9 expression thus induces gene knockout.]

Figure 7. Generation of Puro-iCr donor plasmid
(A) The pEntr-Cr vector contains the chimeric gRNA expression module and Cas9 expression cassette. (B) Cloning of the target DNA sequence into the pEntr-Cr vector via digestion with BbsI and ligation. Note: Due to the requirement of U6 promoter to have a ‘G’ base at the transcription start site, one base ‘G’ followed by 19 Ns is cloned into the pEntr-Cr backbone. (C) Through GATEWAY LR reaction, the chimeric gRNA expression module and Cas9 expression cassette in the pEntr-cr is transferred to Puro-iDEST to generate Puro-iCr donor plasmid.]