A CRISPR/Cas-Mediated Selection-free Knockin Strategy in Human Embryonic Stem Cells

Zengrong Zhu, Nipun Verma, Federico González, Zhong-Dong Shi, and Danwei Huangfu

1Developmental Biology Program, Sloan Kettering Institute, 1275 York Avenue, New York, NY 10065, USA
2Weill Graduate School of Medical Sciences at Cornell University/The Rockefeller University/Sloan Kettering Institute Tri-Institutional M.D.-Ph.D. Program, 1300 York Avenue, New York, NY 10065, USA
3Co-first author

*Correspondence: huangfu@mskcc.org
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SUMMARY

The development of new gene-editing tools, in particular the CRISPR/Cas system, has greatly facilitated site-specific mutagenesis in human embryonic stem cells (hESCs), including the introduction or correction of patient-specific mutations for disease modeling. However, integration of a reporter gene into an endogenous locus in hESCs still requires a lengthy and laborious two-step strategy that involves first drug selection to identify correctly targeted clones and then excision of the drug-resistance cassette. Through the use of iCRISPR, an efficient gene-editing platform we recently developed, this study demonstrates a knockin strategy without drug selection for both active and silent genes in hESCs. Lineage-specific hESC reporter lines are useful for real-time monitoring of cell-fate decisions and lineage tracing, as well as enrichment of specific cell populations during hESC differentiation. Thus, this selection-free knockin strategy is expected to greatly facilitate the use of hESCs for developmental studies, disease modeling, and cell-replacement therapy.

INTRODUCTION

Human embryonic stem cells (hESCs) are capable of unlimited self-renewal in culture while maintaining the potential to differentiate into any cell type present in the human body and thus provide researchers great opportunities for human developmental studies, disease modeling, and cell-replacement therapies (Zhu and Huangfu, 2013). All these applications benefit from lineage-specific knockin reporters that allow real-time observation of gene-expression dynamics, cell-lineage tracing, and isolation of a specific cell population of interest from a heterogeneous differentiation culture for downstream analysis. However, creating knockin alleles in hESCs is usually a lengthy and technically challenging process. Because of the low efficiency of homologous recombination, the donor vector needs to contain a drug-resistance gene for enrichment of cells with the correct integration. Due to the concern that the insertion of a drug-resistance cassette may interfere with the expression of the reporter gene or neighboring genes, it is usually necessary to remove the drug-resistance cassette through a second electroporation step followed by isolation of clonal lines and further characterization (Davis et al., 2008). Thus, substantial time and effort is needed to generate a knockin reporter hESC line through this two-step procedure.

The development of engineered “genomic scissors” that introduce site-specific DNA double-strand breaks (DSBs), including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and more recently the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, has greatly facilitated gene targeting in hESCs (Kim and Kim, 2014). Repair of a DSB by non-homologous end joining (NHEJ) often results in insertion and/or deletions (Indels) that can be used to knock out a target gene in hESCs (Ding et al., 2013a, b; Gonzalez et al., 2014). Alternatively, homology-directed repair (HDR) can be employed to efficiently incorporate exogenous sequences such as a fluorescent reporter into a specific genomic locus in hESCs (Hockemeyer et al., 2009, 2011; Hou et al., 2013; Merkert et al., 2014). Despite the significant improvement, a drug-resistance cassette is still required for generating knockin reporters of genes that are not expressed in undifferentiated hESCs.

To overcome these limitations, we made use of the CRISPR/Cas system, in which the CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) duplex or a single chimeric guide RNA (gRNA) recognizes a 20-nucleotide (nt) DNA sequence upstream of the 5'-NGG-3' protospacer adjacent motif (PAM) and directs the DNA endonuclease Cas9 for site-specific cleavage (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013a). Based on this, we have developed an efficient genome-editing platform in hESCs, which we named iCRISPR (Gonzalez et al., 2014). Through TALEN-mediated gene targeting in the AAVSTi locus, we have created hESC lines (referred to as iCas9 hESCs) that allow robust, doxycycline-inducible expression of Cas9. By transfecting iCas9 hESCs with gRNAs, the iCRISPR system enables efficient NHEJ-mediated gene disruption as well as HDR-mediated precise nucleotide modifications in the presence of short single-stranded DNA (ssDNA) donors (~100 nt).

We reasoned that the iCRISPR system would also facilitate the generation of knockin reporter alleles using longer
double-stranded (dsDNA) donors and may further enable the identification of correctly targeted hESC lines without drug selection. Thus, this work explores the utility of iCRISPR for targeting fluorescent reporters into two endogenous loci, OCT4 (POU5F1) and PDX1, and demonstrates the generation of knockin hESC lines without drug selection for both expressed and silent genes. Further characterization confirmed the creation of multiple hESC reporter lines with no undesired mutations in the targeted loci or any potential off-target sites analyzed, supporting the broad application of this approach for efficient generation of knockin alleles in hESCs.

RESULTS

CRISPR/Cas-Mediated Targeting of the OCT4 Locus

We first evaluated the efficiency of the CRISPR/Cas system for making knockin reporter alleles by targeting the OCT4 locus using drug selection. HUES8 hESCs were co-electroporated with two plasmids: one expressing Cas9 and a crRNA/tracrRNA duplex targeting OCT4 and the other containing the fluorescent reporter and a drug-resistance cassette as the HDR template (Figures 1A, S1A, and S1B). We used a donor plasmid, 2A-eGFP-PGK-Puro (Hockemeyer et al., 2011), in which the last OCT4 coding codon is fused in frame with a 2A sequence followed by eGFP (2A-eGFP) and a loxP-flanked (floxed) puromycin-resistance gene expressed from the constitutive PGK promoter (PGK-Puro) (Figure 1A). This strategy minimizes any potential impact on the endogenous protein and is applicable to targeting both silent and expressed genes. PCR and Southern blot analysis identified eight correctly targeted clones without random transgene integration from a total of 288 puromycin-resistant clones screened (Figures 1B and S1C). The targeting efficiency (2.8%) was comparable to the efficiencies observed with TALENs and ZFNs using similar targeting strategies (Hockemeyer et al., 2009, 2011).

Despite correct targeting, we failed to detect eGFP expression in any of the targeted lines. This is likely caused by the presence of the drug-resistance cassette as observed for other genes (Davis et al., 2008). Indeed, after Cre-mediated excision of the PGK-Puro cassette, all resulting OCT4-eGFP lines showed proper co-expression of eGFP with pluripotency markers OCT4, SOX2, and NANOG (Figures 1C, 1D, and S1D). These results highlight the necessity of removing the drug-resistance cassette for proper reporter gene expression.

Selection-free Targeting of the OCT4 Locus Using a Mini-vector Donor

To further explore the possibility of making knockin reporter alleles without drug selection, we designed a “mini-vector” donor plasmid, 2A-mOrange, which is similar to 2A-eGFP-PGK-Puro except that there is no PGK-Puro cassette and eGFP was replaced by mOrange (Figure 2A). We also replaced the crRNA/tracrRNA duplex cr1-dp with the single gRNA cr1 targeting the same sequence (Figures 2A and S2A), as the chimeric version works more efficiently than the original duplex design (Hsu et al., 2013; Jinek et al., 2012). Similar to the experiment with the 2A-eGFP-PGK-Puro donor, we co-electroporated HUES8 hESCs with a plasmid expressing Cas9/gRNA and the new 2A-mOrange mini-vector (Figure 2A). In contrast to the absence of fluorescence after integration of the 2A-eGFP-PGK-Puro cassette, integration of the 2A-mOrange cassette resulted in mOrange expression in ~0.001% of cells as detected by fluorescence-activated cell sorting (FACS) (Figure S2B). One may enrich mOrange-expressing cells for establishing OCT4 reporter lines. However, this low efficiency is impractical for genes not expressed in undifferentiated hESCs, as one has to rely on randomly picking individual colonies to establish clonal lines.

Our recent study shows that the iCRISPR platform enables efficient gene editing using short ssDNA HDR templates (González et al., 2014), prompting us to further optimize the iCRISPR platform for HDR using longer circular dsDNA donor vectors. After optimizing transfection conditions, we co-transfected doxycycline-treated HUES8 iCas9 cells twice in 2 days with the OCT4 cr1 gRNA and the 2A-mOrange mini-vector using Lipofectamine 3000 (Figures S2D and S3). FACS analysis identified ~0.4% mOrange-expressing cells (Figure 2B), >100-fold greater than results from our electroporation experiments (Figures S2B and S2C). Similar results were observed in experiments using iCas9 cells generated from MEL-1 hESCs (Figure S2E), supporting the general utility of this new approach in diverse human pluripotent stem cell (hPSC) backgrounds. The much-improved efficiencies can be partially attributed to the integration of Cas9 in the genome as an ~5- to 6-fold increase of mOrange+ cells was observed compared to the control condition where iCas9 hESCs (not treated with doxycycline) were transfected with Cas9/gRNA and the donor vector using Lipofectamine 3000 (Figure 2C). The use of Lipofectamine transfection also substantially increased the targeting efficiency compared to the electroporation method (Figures S2B and S2C). There may be other ways to improve the transfection efficiency (e.g., through nucleofection) to achieve similar results with or without the use of iCas9 hESCs (Byrne et al., 2015).

We picked ten colonies from individual FACS-isolated mOrange+ cells and identified six correctly targeted clones by PCR and Southern blot analysis (Figures 2B, 2D, and S2F). All six lines co-expressed mOrange with pluripotency markers such as OCT4, SOX2, and NANOG and displayed normal hESC morphology (Figure S2G). We further
examined the OCT4-mOrange hESC reporter lines along with the OCT4-eGFP lines for reporter gene expression after differentiation. After 3 days of treatment with BMP4 and SB431542, a TGFβ inhibitor (Hou et al., 2013), hESCs exhibited a differentiated morphology, and eGFP and mOrange expression were downregulated in the respective OCT4-eGFP and OCT4-mOrange hESC reporter lines with concomitant loss of endogenous OCT4 expression as determined by immunostaining and FACS analysis (Figures 3A and 3B). Thus, the OCT4-eGFP and OCT4-mOrange reporters faithfully reflect endogenous gene expression during the maintenance and differentiation of hESCs.

We next investigated whether the relatively high targeting efficiency was achieved at the expense of undesirable

Figure 1. CRISPR/Cas-Mediated Targeting of the OCT4 Locus through Drug Selection
(A) Schematics of the targeting strategy. In the presence of the donor plasmid, HDR results in the replacement of the OCT4 stop codon with 2A-eGFP-PGK-Puro. The PCR primers (F + GFP-R) used for genotyping are indicated with red arrowheads. OCT4 cr1-dp (the duplex version) targets a 30-nt sequence (indicated with a green line) upstream of the PAM sequence (indicated with a purple line). In all targeting schematics here and after, boxes are exons, filled blue boxes indicate the coding sequence (CDS), connecting lines are introns, the stop codon (TGA) is labeled in red, HL and HR indicate left and right homology arms, and the Southern blot external and internal probes are indicated with red bars.

(B) Southern blot analysis using the external probe (WT: 4,173 bp; GFP+Puro: 6,835 bp) and the internal puromycin probe (GFP+Puro: 2,415 bp) identified eight correctly targeted clones, which are labeled in red. WT, wild-type allele; GFP+Puro, correctly targeted allele with puromycin-selection cassette.

(C) Three of the correctly targeted clones (nos. 1, 4, and 7) were electroporated with Cre recombinase. Four days after electroporation, eGFP+ cells were isolated using FACS.

(D) For each clone (nos. 1, 4, and 7) electroporated with CRE recombinase, two GFP+ clones were picked (e.g., C1.1 and C1.2 for clones derived from no. 1), and Southern blot analysis using the external probe (WT: 4,173 bp; GFP+Puro: 6,835 bp; GFP only: 5,015 bp) and the internal puromycin probe (GFP+Puro: 2,415 bp) showed correct removal of the puromycin-selection cassette. A clone (C1) prior to Cre electroporation was used as the Pre-Cre control. GFP+Puro, targeted allele prior to Cre-mediated excision of the PGK-Puro cassette; GFP only, targeted allele after Cre-mediated excision.
mutations at the OCT4 locus or any off-target sites. All eight OCT4-eGFP and six OCT4-mOrange lines examined showed the expected sequence at the junction between the endogenous OCT4 sequence and the inserted sequence. This is reassuring, as we made sure that the donor template did not contain the CRISPR target sequence to prevent undesired mutagenesis after reporter gene integration. However, Indel mutations were detected in the non-targeted allele in two of the six OCT4-mOrange reporter lines examined (Figure 3C). These findings underscore the necessity of thorough sequence analysis for eliminating clones with undesired mutations in the non-targeted allele, a point not widely recognized with the CRISPR/Cas-mediated targeting strategy. We also sequenced seven predicted off-target sites based on the 12-bp seed sequence important for target recognition (Jiang et al., 2013; Jinek et al., 2012). Examination of six OCT4-mOrange and eight OCT4-eGFP lines revealed no mutations except that three OCT4-eGFP lines carried mutations at the POU5F1P4 locus, which shares the same 20-nt target sequence with the intended target (Table S1).

Targeting the PDX1 Locus Using a Mini-vector Donor without Drug Selection
We further investigated whether this selection-free approach also applied to genes not expressed in undifferentiated hESCs. We chose to target PDX1, which encodes a transcription factor not expressed in undifferentiated hESCs but in pancreatic progenitors and their differentiated progeny such as pancreatic β cells. The ability to monitor PDX1 expression during in vitro differentiation and to enrich PDX1+ pancreatic progenitor cells or β cells...
would be valuable for studies of pancreatic development and the use of hESCs for β cell replacement therapies.

We designed two gRNAs (PDX1 cr1 and cr2) to target DNA sequences in proximity to the PDX1 stop codon and used a PDX1-eGFP mini-vector donor for integration of the eGFP reporter into the PDX1 locus (Figures 4A and S4A). Correct targeting should enable expression of eGFP from the endogenous PDX1 locus with minimal impact on PDX1 protein expression or function. Following establishment of clonal lines (Figure 4B), PCR and Southern blot analysis identified three correctly targeted clones without random integration: two clones with eGFP
integration in one PDX1 allele and one clone with biallelic integration (Figures 4C, 4D, and S4B). All three PDX1-eGFP reporter lines displayed normal hESC morphology and expressed pluripotency markers OCT4, NANOG, and SOX2 as determined by immunofluorescence staining (Figure S4C). After differentiation into pancreatic progenitors, co-expression of GFP and PDX1 was observed, demonstrating faithful reporter activity (Figure 4E). Thus, we successfully generated multiple faithful knockin reporter lines for PDX1, a gene with a lineage-restricted expression pattern in differentiated hESCs.

We further examined the PDX1 reporter lines for potential undesired mutations similar to analysis performed on the OCT4 reporter lines. We found the expected sequences in both the targeted and non-targeted PDX1 allele in all clones (Figure 4F) except for one (no. 3) with a 24-bp deletion in the non-targeted allele. Recent studies suggest that CRISPR/Cas9 tolerates mismatches between CRISPR and DNA sequences.

Figure 4. Generation of PDX1-eGFP Reporter Lines without Drug Selection
(A) PDX1 cr1 and cr2 were designed to target sequences in proximity to the PDX1 stop codon. In the presence of the eGFP donor plasmid, HDR resulted in the replacement of the stop codon with 2A-eGFP. PCR primers (F + R) used for genotyping are indicated with red arrowheads.
(B) Timeline for the generation of PDX1-eGFP hESC lines using the iCRISPR platform. DOX, doxycycline.
(C) PCR genotyping of 12 clones, identified from the PCR screen in Figure S4B, that showed the correct PCR product for the targeted allele (GFP: 1,746 bp).
(D) Southern blot analysis using the external probe and internal probe (WT: 3,845 bp; GFP: 4,632 bp) identified three correctly targeted lines. Lines without random integrations and carrying mono-allelic eGFP insertion are labeled in red, and the clone with a bi-allelic eGFP insertion is labeled in green.
(E) Immunofluorescence staining displayed co-expression of GFP and PDX1 in pancreatic progenitors differentiated from PDX1-eGFP hESCs. The scale bar represents 100 μm.
(F) Sanger sequencing of non-targeted and targeted PDX1 alleles showed correct targeting of the PDX1 locus with no undesired mutations.
target DNA at different positions in a sequence-dependent manner, influenced by the number, position, and distribution of mismatches (Hsu et al., 2013; Tan et al., 2015). Using a prediction program developed by Feng Zhang’s group (http://crispr.mit.edu), we sequenced 20 most likely off-target sites (ten each predicted for PDX1 cr1 and cr2) and detected no mutations (Table S1).

**DISCUSSION**

Here, we demonstrate the generation of hESC reporter lines without the use of drug selection for both active and silent genes through the use of the iCRISPR system. Selection-free gene targeting eliminates the need for removal of drug-resistance cassette after identification of correctly targeted clones, and mini-vector donors with short homology arms (~500–1,000 bp each) are convenient to make. Thus, this method significantly reduces the time and effort required for establishing hESC reporter lines. Additionally, conventional gene-targeting strategies typically use the Cre-loxP strategy to remove drug-resistance cassettes, which leaves behind a 34-bp loxP “scar” in the endogenous locus. Although not an issue in most cases, this residual sequence could interfere with the expression of the targeted gene in some situations (Meier et al., 2010). In comparison, our strategy eliminates the need for the selection cassette and thereby minimizes the alteration of the endogenous locus. Although we focused on creating promoter-fusion reporters, the same knockin approach can be readily applied to making protein-fusion reporters for visualizing protein subcellular localization, precisely deleting or replacing specific genomic sequences, and introducing or correcting disease-associated mutations.

Previously, we and others have failed to target the PDX1 locus using traditional targeting approaches (Z.Z. and D.H., data not shown; E. Stanley, personal communication), yet the absolute targeting efficiencies using the selection-free method were comparable between the PDX1 and OCT4 loci. It is known that gene-targeting efficiencies can vary significantly depending on the target locus, though the exact reason is unclear. Traditional gene targeting relies on drug selection; thus, the relative targeting efficiencies after drug selection depend, at least in part, on the expression of the drug-resistance gene from the targeted locus. Because the expression of drug-resistance gene may differ significantly between expressed and silent loci, the relative targeting efficiency after drug selection for a lineage-specific gene may appear much lower compared with a pluripotency gene. For certain loci, the drug-resistance gene may be expressed at such low levels that hinder the identification of a correctly targeted clone using the drug-selection method (Rostovskaya et al., 2012). Because our targeting approach obviates drug selection, it may overcome such bias and facilitate the generation of reporter alleles for genes that were previously difficult to target. One may further use this ability to measure absolute targeting frequencies to compare HDR efficiencies across different genomic contexts.

There have been concerns about potential off-target mutagenesis with the CRISPR/Cas system (Cho et al., 2014; Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013b; Pattanayak et al., 2013). Our analysis so far did not reveal any off-target mutations at sites without perfect complementarity with the CRISPR target sequence. However, we cannot exclude the possibility of off-target mutations elsewhere in the genome, and a thorough analysis may be necessary before the reporter lines are used in future studies. The CRISPR/Cas system is continuously improved with the development of better algorithms for CRISPR design and off-target prediction. It is reassuring that a recent high-coverage whole-genome sequencing study failed to detect significant incidence of off-target mutations in CRISPR-targeted hPSC lines (Veres et al., 2014). On the other hand, we noticed that some correctly targeted clones carried mutations in the non-targeted allele, though the targeting efficiency is sufficiently high that one could simply discard the minority of clones carrying mutations. One may also target intronic regions with low-sequence conservations to further mitigate any concerns associated with Indel mutations in the non-targeted allele.

Our selection-free targeting approach enables rapid generation of knockin reporter lines, though it also requires either using established iCas9 cells or creating new iCas9 lines in a desired hPSC background. The upfront effort for generating iCas9 cells is relatively small due to the efficient TALEN-mediated AAVS1-targeting approach, and it is possible to establish an iCas9 line in about 1 month (González et al., 2014; Zhu et al., 2014). Once an iCas9 line is established, it can be used for making different types of reporters. Our previous study has shown that Cas9 activity is tightly regulated by doxycycline treatment, and established iCas9 lines exhibit no apparent chromosomal aberrations or defects in the maintenance of the pluripotent state (González et al., 2014). A recent study also observed no adverse effects in constitutive Cas9-expressing mice (Platt et al., 2014). An additional benefit of using iCas9 hPSCs for making reporter lines is that the cells can be conveniently used for a variety of downstream genetic studies using gene-editing approaches we already established (González et al., 2014). Thus, we expect this selection-free knockin strategy to further facilitate the use of hESCs for developmental studies, disease modeling, and cell-replacement therapy.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.04.016.

AUTHOR CONTRIBUTIONS

D.H., Z.Z., N.V., and F.G. conceived the project; Z.Z. performed most experiments related to PDX1 targeting; N.V. performed most experiments related to OCT4 targeting; F.G. and Z.-D.S. performed additional relevant experiments; and D.H., Z.Z., and N.V. wrote the manuscript.

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**Figure S1**

**A**

![Diagram of CRISPR-Cas9 system](Image)

**B**

| CRISPRs | CTRL | #1 | #2 | #3 | #4 |
|---------|------|----|----|----|----|
| U6      | U    | U  | D  | D  | U  |
| crRNA   | D    | D  | U  | D  | U  |
| NLS     | U    | U  | U  | U  | U  |
| Cas9    | D    | D  | D  | D  | D  |
| U6      | D    | D  | U  | D  | U  |
| pA      | U    | U  | U  | U  | U  |
| pUC ori | D    | D  | U  | D  | U  |

- Indel (%): 49
- 46
- 38

**C**

**PCR Genotyping**

*Colonies 1-96*

*Colonies 97-192*

*Colonies 193-288*

**D**

**GFP/OCT4**

**OCT4**

**SOX2**

**GFP**

**NANOG**

**Brightfield**
Figure S2

A) piCRg Entry

B) HUES8 electroporation

C) HUES8 iCas9 -DOX, electroporation

D) iCas9 with gRNA+donor co-transfection

E) MEL-1 iCas9

F) PCR genotyping

G) Brightfield | DAPI | OCT4 | mOrange | OCT4/mOrange

| Brightfield | DAPI | NANOG | SOX2 | NANOG/SOX2 |
Figure S3

A

|                    | None-Transfected | Electroporation | JetPrime |
|--------------------|------------------|-----------------|---------|
| Alexa 555 dsRNA    | 0.346            | 19.3            | 61.1    |
| Alex 555 dsRNA     | 99.5             | 75.6            | 19.9    |
| GFP plasmid        | 0.127            | 3.3             | 0.64    |

Lipo RNAiMax

|                    | 78.9            | 72.4            | 60.8    |
|--------------------|-----------------|-----------------|---------|
| Alexa 555 dsRNA    | 7.64            | 19.5            | 20      |
| GFP plasmid        | 13.4            | 7.99            | 16.8    |
|                   | 0.0785          | 0.097           | 2.36    |

Lipo 2000

|                    | 19.3            | 19.5            | 61.1    |
|--------------------|-----------------|-----------------|---------|
| Alexa 555 dsRNA    | 19.5            | 75.6            | 19.9    |
| GFP plasmid        | 19.5            | 3.3             | 0.64    |
|                   | 7.64            | 7.99            | 2.36    |

Lipo 3000

|                    | 61.1            | 72.4            | 60.8    |
|--------------------|-----------------|-----------------|---------|
| Alexa 555 dsRNA    | 18.4            | 19.5            | 20      |
| GFP plasmid        | 16.8            | 7.99            | 16.8    |
|                   | 0.64            | 0.097           | 2.36    |

B

None-Transfected

Electroporation

JetPrime

GFP/Brightfield

Lipo RNAiMax

Lipo 2000

Lipo 3000
Figure S4

A

d-1 d0 d1 d2 d3

DOX

gRNA+donor

do + + + +
donor - + + +
gRNA - - cr1 cr2

B

PCR Genotyping

PDX1 cr1

PDX1 cr2

C

PDX1eGFP/+ PDX1eGFP/eGFP

OCT4/NANOG/Sox2

Brightfield

PDX1eGFP/+ PDX1eGFP/eGFP
**Figure S1. CRISPR-mediated targeting of the OCT4 locus through drug selection.**

(A) The vector map of the px260 plasmid for expressing Cas9 and crRNA/tracrRNA. (B) Four crRNAs were designed to target the stop codon of the OCT4 locus. Each crRNA was cloned into the px260 vector and transfected into 293T cells. Two days after transfection genomic DNA was collected and Surveyor analysis was used to estimate the efficiency of each CRISPR in generating Indels. Asterisks indicated the cleavage products and the estimated Indel frequencies were labeled in blue. CTRL: px260 vector control; U: undigested control; D: digestion reaction with Surveyor nuclease. (C) PCR genotyping results showing 10 positive clones (indicated by red asterisks) identified based on the presence of a correct PCR product (811 bp). (D) OCT4-eGFP reporter hESCs were stained for pluripotency markers OCT4, NANOG and SOX2, which overlapped with GFP expression. The GFP signal was detected using a GFP antibody. Scale bar = 100 µm.

**Figure S2. Targeting the OCT4 locus without drug selection.**

(A) The vector map of the piCRg Entry plasmid for expressing Cas9 and the chimeric gRNA. (B) FACS analysis for OCT4-mOrange expressing cells in HUES8 cells after electroporation of the OCT4-mOrange donor and the Cas9/gRNA plasmids. (C) FACS analysis for OCT4-mOrange expressing cells in HUES8 iCas9 cells without doxycycline treatment after electroporation of the OCT4-mOrange donor and the Cas9/gRNA plasmids. (D) Timeline for establishing hESC reporter lines using iCas9 hESCs. (E) FACS analysis for OCT4-mOrange expressing cells in MEL-1 iCas9 cells treated with doxycycline and transfected with the OCT4-mOrange plasmid and OCT4 cr1 gRNA. (F) PCR genotyping for OCT4-mOrange gene targeting. Correctly targeted clones are
indicated in red. (G) OCT4-\textit{mOrange} reporter hESCs were stained for pluripotency markers OCT4, NANOG and SOX2, which overlapped with \textit{mOrange} expression. Scale bar = 100 µm.

**Figure S3. Optimizing co-transfection of DNA and RNA into hESCs.**

(A) FACS analysis of hESCs co-transfected with Alexa 555 dsRNA and a GFP-expressing plasmid (~ 10 kb) using four commonly used transfection reagents along with electroporation. Lipofectamine 3000 consistently performed better than the other conditions. Although comparable efficiencies could sometimes be achieved using JetPrime (as shown here), the outcomes were variable and appeared to relate to the amount of DNA used for transfection. (B) Stronger GFP expression was detected in hESCs transfected with Lipofectamine 3000, suggesting that increased copy number of GFP-expressing plasmid was transfected into the cell.

**Figure S4. Generation of PDX1-eGFP reporter hESCs without drug selection.**

(A) Population level PCR analysis of HUES8 iCas9 cells two days after transfection with gRNA and donor plasmid. (B) PCR genotyping of replated clonal hESCs after transfection. Correctly targeted cells are labeled with a red asterisk. (C) \textit{PDX1-eGFP} reporter hESC lines displayed proper expression of pluripotency markers OCT4, NANOG and SOX2 and typical hESC morphology. The brightfield images were taken from live cells, and do not correspond to the immunofluorescence images of fixed cells.
### Table S1. Off-target analysis

Sequencing analysis of potential off-target sites in OCT4-eGFP and OCT4-mOrange reporter lines

| Gene         | CRISPR Target Sequence-PAM | Sequencing results in OCT4-eGFP reporter lines | Sequencing results in OCT4-mOrange reporter lines |
|--------------|----------------------------|-----------------------------------------------|-------------------------------------------------|
| OCT4 (intended target) | TCTCCCATGCATTCAAACCTG–AGG | 1: WT  2: WT  4: WT  5: WT  6: WT  7: WT  9: WT  10: WT | 3: WT  5: WT  6: WT  7: WT  8: WT  10: WT |
| DLG2         | AAGCTCAGGCAATTCAAACCTG–TGG | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |
| GPHN         | GCCCTAGGCAATTCAAACCTG–TGG | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |
| IMMP2L       | TAGACTTACATCAAACCTG–AGG   | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |
| PEMT         | GCACCTAGGCAATTCAAACCTG–TGG| WT    WT    WT    WT    WT    WT    WT    WT    WT    WT | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |
| POLR2J4      | AAGGAGAAGGCAATTCAAACCTG–TGG| WT    WT    WT    WT    WT    WT    WT    WT    WT    WT | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |
| POU5F1P4     | TCTCCCAGGCAATTCAAACCTG–AGG| WT    HET*   WT    HOM**  WT    HOM*** WT    WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |
| SLC33A1      | CAAATGGCAATTCAAACCTG–CGG | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT | WT    WT    WT    WT    WT    WT    WT    WT    WT    WT |

*WT: Both alleles are wild-type; HET: One allele has a mutation; HOM: Both alleles have mutations; 6 bp insertion; 4 bp deletion; 10 bp deletion.

Sequencing analysis of potential off-target sites in PDX1-GFP reporter line #9 generated using PDX1 cr1

| Gene         | CRISPR Target Sequence-PAM | Sequencing results in PDX1-GFP reporter line #9 |
|--------------|----------------------------|-----------------------------------------------|
| PDX1 (intended target) |GCCGCAGGAACCACGATGAG–AGG | WT    |
| SUSD4        | TCTCAGGCAACCAGATGG–CAG   | WT    |
| COX7C        | TCGTAGGACCCACGATGG–GAG   | WT    |
| DCPS         | GCCACAGCCAACGATGG–GAG    | WT    |
| TRAF3IP2     | GCCCTGGGACAGGCAAGAG–AGG | WT    |
| NARF         | GCCGCAGGACCCAGGAGAAG–CGG| WT    |
| ILVBL        | CGACGCAAACCAGGAGAG–AGG  | WT    |
| BST2         | CGCGCAGGAGCAAGAGAGAG–AGG| WT    |
| SNED1        | CGCGCAGGACCCGAGGAGGAG–AGG| WT    |
| PSG3         | CCTGACAGGAAACCAGGATAAG–AGG| WT    |
| CRAT         | GGGGCAGGAACCAGCTGAG–TGG | WT    |
Sequencing analysis of potential off-target sites in *PDX1-GFP* reporter line #4 generated using *PDX1 cr2*

| Gene         | CRISPR Target Sequence-PAM | Sequencing results in *PDX1-GFP* reporter line #4 |
|--------------|-----------------------------|---------------------------------------------------|
| *PDX1* (intended target) | GCCTCTCATCGTGGTTCCTG-CGG   | WT                                                |
| *PSG3*       | TCCTTTATCCTGTTCCCTG-CAG     | WT                                                |
| *NUBP1*      | GCATCCACGTGTTCCCTG-AGG      | WT                                                |
| *SYNM*       | GTGTCTGACCGTGGTCCCTG-GAG    | WT                                                |
| *HEXDC*      | GCCACTGCACGTGTTCCCTG-AGG    | WT                                                |
| *PSG10P*     | TTCTTTATCCTGTTCCCTG-CAG     | WT                                                |
| *KCNQ1*      | TGCTCTACCTTGGTCCCTG-GGG     | WT                                                |
| *FBRSL1*     | GCCAGGCACGCGGTTCCCTG-CAG    | WT                                                |
| *FOXJ3*      | ACCTGCCCATGTTGTTCCCTG-GAG  | WT                                                |
| *OR6M1*      | ATCTCTCTCGTGTTCTGTG-TGG     | WT                                                |
| *MAGEA8*     | ACCTCACAGCTGTTCCCTG-GAG     | WT                                                |
**Table S2. Oligonucleotides used in this study**

**Oligonucleotides for generating Cas9/crRNA/tracrRNA or Cas9/gRNA expressing plasmids**

| Gene | CRISPR target sequence (5' of PAM) | Vector | Oligos used for generating the CRISPR constructs (5' to 3') |
|------|----------------------------------|--------|--------------------------------------------------------|
| **OCT4** | | | |
| cr1-dp | CACTCTGGGTCTCTCCCATGCATTCAAACTG | px260 | F: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG<br>R: TAAAACCAGTTTGATGCGATGGAGGAGGCCAGAGTG |
| cr2-dp | TGCATTCAAACTGAGGTGCCCTGCTCCCTCTTA | px260 | F: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG<br>R: TAAAACCAGTTTGATGCGATGGAGGAGGCCAGAGTG |
| cr3-dp | ATGCATTCAAACTGAGGTGCCCTGCTCCCTCTCT | px260 | F: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG<br>R: TAAAACCAGTTTGATGCGATGGAGGAGGCCAGAGTG |
| cr4-dp | CTCTGGGTCTCTCCCATGCATTCAAACGTG | px260 | F: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG<br>R: TAAAACCAGTTTGATGCGATGGAGGAGGCCAGAGTG |
| cr1 | TCTCCCATGCATTCAAACGTG | piCRg Entry | F: CACGGCCATCCATGCATTCAAACGTG<br>R: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG |
| **PDX1** | | | |
| cr1 | GCCGCAGGAACCACGATGAG | piCRg Entry | F: CACGGCCATCCATGCATTCAAACGTG<br>R: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG |
| cr2 | GCCTCTCATCGTGGTTCCTG | piCRg Entry | F: CACGGCCATCCATGCATTCAAACGTG<br>R: AAACCACTCTGGGTCTCTCCCATGCATTCAAACGTG |

**PCR Primers for generating templates for gRNA in vitro transcription**

| Gene | CRISPR target sequence (5' of PAM) | CRISPR specific forward primer (5' to 3') |
|------|----------------------------------|----------------------------------------|
| **OCT4** | | |
| cr1 | TCTCCCATGCATTCAAACGTG | F: TAATACGACTCTATAGGGTCTCTCCCATTTCATCAAACGTG |
| **PDX1** | | |
| cr1 | GCCGCAGGAACCACGATGAG | F: TAATACGACTCTATAGGGTCTCTCCCATTTCATCAAACGTG |
| cr2 | GCCTCTCATCGTGGTTCCTG | F: TAATACGACTCTATAGGGTCTCTCCCATTTCATCAAACGTG |

**Universal reverse primer**

gRNA-R: AAAAGCACCAGACTCTGGTGGCC
### PCR Primers for donor plasmid construction

| Donor plasmid | Primer sequence (5' to 3') |
|---------------|----------------------------|
| OCT4          | **Nh2AOr-F** TTCTAGCTAGCACCAGTGCCACGAACGTCTCTCTCTGGTTAAAGCAAGCAGGAGAGCGTGGAGAAGAAAGCCCGG<br>TCCTAGTGAGCAGGAGGAGGGAGG |
|               | **AscOr-R** CTTATGGCGCGCTCGGAACCTTGAGCTACAGCTTGCCAT |
| PDX1-eGFP     | **HL-F** GTCAGTCTGAGCAGCCACACTGCATTGGGAGAGG<br>**HL-R** GTCAGTCTGAGCAGCCACACTGCATTGGGAGAGG<br>**HR-F** GTCAGTCTGAGCAGCCACACTGCATTGGGAGAGG<br>**HR-R** GTCAGTCTGAGCAGCCACACTGCATTGGGAGAGG<br>**2A-GFP-F** CAGTGCTAGGCGCCACTAAGTTCCCTTGTT<br>**2A-GFP-R** TCACTCGCGGGCTTACTTGAGCTCGTCCA |

### PCR primers for genotyping

| Gene | Primer sequence (5' to 3') |
|------|----------------------------|
| OCT4 | **F** AGTCCAAAGCTTGCCCTTGTCACC<br>**GFP-R** AGCTCCCTGAGCAGGTCACACC<br>**mOr-R** GAGGTGATGCTCAACTTGATGCGGA |
| PDX1 | **F** ATTTGCTGGCTCTCAGGTTG<br>**R** GTTCACCTTGATGCGGCTTT |

### PCR primers for generating Southern blot probes

| Gene | Probe | Primer sequence (5' to 3') |
|------|-------|----------------------------|
| OCT4 | External | **F** CCAGTGGAGTCACTGGGCT<br>**R** GTCCGACTCCCAAGAGGTCACACC |
|      | Internal Puro | **F** TGACCCAGATACAGGCGCCACCG<br>**R** TCGTAAAGGGAGTTGGC |
|      | Internal mOrange | **F** TGCCCATCATTGACAGAGTCTCA<br>**R** CTCTTTCTGGATTACGGCCCCG |
| PDX1 | External | **F** AACACCTGCTGGGCAATTTG<br>**R** CGGACACTGCAGGTCAGGTT |
|      | Internal GFP | **F** GTCATCTGCACCACCCCGG<br>**R** CGCGTTTCTCGTTGGG |
Supplemental Experimental Procedures

Generation of constructs

To generate CRISPR plasmids expressing Cas9 and the crRNA/tracrRNA duplex targeting specific genomic loci, 30-bp protospacer sequences were cloned into the px260 (Addgene: 42229) as previously described (Cong et al., 2013). Briefly, vectors were digested with BbsI, treated with Antarctic Phosphatase, and gel purified. A pair of oligonucleotides containing the 30-bp protospacer sequence was annealed generating BbsI overhangs, and cloned into BbsI-digested, dephosphorylated vectors. The same procedure was also used to generate CRISPR plasmids expressing Cas9 and the chimeric gRNA with the difference that a pair of oligonucleotides containing the 20-bp protospacer sequence was cloned into the piCRg Entry plasmid (Addgene: 58904). The sequences for all oligonucleotides used for generating the CRISPR constructs are listed in Table S2.

For generation of OCT4-eGFP hESC reporter lines, the OCT4-2A-eGFP-PGK-Puro plasmid (Addgene: 31938) was used. The left homology arm is 697 bp and the right homology arm is 699 bp. The OCT4-mOrange hESC reporter lines were made using the OCT4-2A-mOrange targeting vector. To generate the OCT4-2A-mOrange targeting vector, an Nhel-2AmOrange-AscI cassette was PCR amplified using the mOrange-pBAD plasmid template (Addgene: 54751) and primers Nh2AOr-F and AscOr-R. Next, the Nhel-2A-mOrange-AscI PCR fragment and the OCT4-2A-eGFP-PGK-Puro plasmid were digested with Nhel and Ascl and ligated.

For constructing the PDX1-GFP donor plasmid, the 725-bp left (Primers: HL-F and HL-R) and 543-bp right (Primers: HR-F and HR-R) homology arms were PCR
amplified from the HUES8 genomic DNA and cloned into the pBlueScript SKII (+) plasmid to generate the pBS-PDX1 plasmid. The 2A-eGFP insert was PCR amplified from the OCT4-2A-eGFP-PGK-Puro plasmid (Addgene: 31938) using 2A-eGFP-F and 2A-eGFP-R primers, digested and cloned into the pBS-PDX1 plasmid to generate the PDX1-eGFP donor plasmid. In the donor plasmid, the 2A-eGFP sequences was fused in-frame to the last codon of *PDX1*. The sequences for all primers used for generating the donor plasmids are listed in Table S2.

**Cell Culture**

HUES8 (NIHhESC-09-0021) and MEL-1 (NIHhESC-11-0139) hESCs were cultured on irradiated mouse embryonic fibroblasts (iMEFs) feeder layers in DMEM/F12 medium (Life Technologies) supplemented with 20% KnockOut Serum Replacement (Life Technologies), 1X MEM Non-Essential Amino Acids (Life Technologies), 1X GlutaMAX (Life Technologies), 100U/ml Penicillin and 100 µg/ml Streptomycin (Gemini), 0.055 mM 2-mercaptoethanol (Life Technologies) and 10 ng/ml recombinant human basic FGF (EMD Millipore). Cells were incubated at 37 °C with 5% (vol/vol) CO₂, and media was changed daily. Cultures were passaged at a 1:6 - 1:12 split ratio every 4 - 6 days using 0.05% trypsin/EDTA. 5 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleck Chemicals) was added into the culture medium when passaging or thawing cells.

293T cells were maintained in DMEM supplemented with 15% fetal bovine serum, 1X GlutaMAX, 1X MEM NEAA and 1mM Sodium Pyruvate (Life Technologies).
Differentiation of hESCs

For differentiation of OCT4 reporter lines, undifferentiated hESCs cultured on iMEF feeder layer were first adapted to the feeder-free E8 culture. Briefly, hESCs were passaged using 0.05% trypsin/EDTA and plated at a 1:3 split ratio on Matrigel (BD Biosciences) coated plates in E8 medium (Stem Cell Technologies). Cells were incubated at 37 °C with 5% (vol/vol) CO2, and media was changed daily. After 2 passages in E8 medium, the cells were differentiated: 2 days after passaging, cells were treated with 10 μM SB431542 (Sigma Aldrich) and 10 ng/mL BMP4 (R&D Systems) to initiate differentiation. After 3 days of this treatment, eGFP and mOrange fluorescence was analyzed by flow cytometry and fluorescence microscopy.

For differentiation into pancreatic lineages, undifferentiated hESC were cultured until 50-70% confluency, washed with PBS w/Ca^{2+}Mg^{2+} once and treated with 2 μM BIO-acetoxyime (Tocris), 100 ng/ml Activin A (R&D) in advanced RPMI (Invitrogen) for 1 day. The medium was changed to 100 ng/ml Activin A in advanced RPMI with 0.2% FBS for 2 days. On day 3, cells were treated with 50 ng/ml FGF10 (R&D) and 0.25 μM SANT1 (Sigma) in advanced RPMI with 2% FBS for 2 days. On day 5, cells were treated with 50 ng/ml FGF10, 0.25 μM SANT1, 2 μM Retinoic Acid (Sigma) and 250 nM LDN (Stemgent) in DMEM with 1% B27 (Invitrogen) for 4 days. Then on day 9, cells were treated with 1 μM Alk5 inhibitor (Axxora), 100 ng/mL Noggin (R&D system) and 1 μM DAPT (Tocris) in DMEM with 1% B27 for 4 days. After that, cells were kept in DMEM with 1% B27 for another 4 days and were examined for the expression of pancreatic cell specific markers.
**In vitro transcription of gRNAs**

A T7 promoter was added to gRNA templates by PCR amplification on piCRg Entry vectors using CRISPR-specific forward primers and a universal reverse primer gRNA-R (Table S2). T7-gRNA PCR products were used as templates for in vitro transcription using the MEGAshortscript T7 kit (Life Technologies). The resulting gRNAs were purified using the MEGAclear kit (Life Technologies), eluted in RNase-free water and stored at -80°C until use.

**Electroporation**

HUES8 hESCs were pre-treated with Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Selleck Chemicals) 24 hours before electroporation. On the day of electroporation, hESCs were disassociated into single cells with 0.25% Trypsin/EDTA and filtered through a 40 µM cell strainer to remove cell clumps. 10 million cells were re-suspended in 800 µL cold PBS and mixed with targeting and donor plasmids (10 µg CRISPR targeting plasmid and 40 µg donor plasmid). Cells were electroporated using the Gene Pulser XCell (Bio-Rad) at 250 V, 500 µF in a 0.4 cm Gene Pulser cuvette (Bio-Rad). Cells were recovered and re-plated on irradiated mouse embryonic fibroblast (iMEF) coated plates with ROCK inhibitor.

For the generation of OCT4-eGFP lines, two days after electroporation cells were treated with puromycin (0.5 µg/mL) for three days. Two weeks after electroporation individual colonies were picked and expanded for PCR genotyping, Southern blot analysis and sequencing for the establishment of reporter lines. Three of the correctly targeted clones (#1, #4 and #7) were electroporated with Cre recombinase following the same procedure described above. After electroporation with 50 µg of Cre recombinase
plasmid, the cells were recovered and replated on iMEF with ROCK inhibitor. 3 days after electroporation, GFP-expressing (GFP*) cells were observed. GFP* cells were isolated by FACS and plated at a low density (2,000 cell/10cm dish) for subsequent picking and expansion of individual clones.

**Transfection**

HUUES8 and MEL-1 iCas9 hESCs (Gonzalez et al., 2014) were treated with ROCK inhibitor and doxycycline one day before transfection. For transfection, cells were dissociated using TrypLE (Life Technologies), replated at 200,000 hESCs per well in iMEF-coated 12-well plates and transfected in suspension with gRNAs and donor plasmid. A second transfection was performed 24 hours later. Transfection of the gRNAs and donor plasmid into hESCs was performed using Lipofectamine 3000 (Life Technologies) following manufacturer’s guidelines. For each targeting, gRNAs at a 10 nM final concentration and 5 µg of donor plasmid were used. Lipofectamine 3000 and gRNA + donor plasmid were diluted separately in Opti-MEM (Life Technologies), mixed together, incubated for 5 min, and added drop-wise to cultured hESCs.

**Assessment of Indel mutations using the Surveyor Nuclease Assay**

80-90% confluent 293T cells in 6-well dishes were transfected using JetPrime transfection reagent (Polyplus) following manufacturer’s guidelines. For each well, a total of 2 µg CRISPR plasmid was transfected. After transfection the cells were incubated at 37 °C for 2 days prior to genomic DNA extraction. The Surveyor Kit (Transgenomic) was used to test the efficiency of each crRNA for producing Indels. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer’s protocol. The genomic region flanking the CRISPR target site for each gene was PCR
amplified. For Surveyor analysis 16 µL of PCR product (at 25 ng/µL) was denatured and re-annealed in JumpStart buffer to a total volume of 20 µL using the following protocol: 95 °C, 5 min; 95–85 °C at −2 °C/s; 85–25 °C at −0.1 °C/s; hold at 4 °C. 10 µL of hybridized PCR products was treated with 1 µL of Surveyor Enhancer S and 1 µL of Surveyor Nuclease S at 42°C for 60 minutes. The reaction was stopped by adding 1.2 µL of Stop solution to each tube. Products were then analyzed on 2.5% agarose gel and imaged with a Gel Doc gel imaging system (Bio-Rad). Quantification was based on relative band intensities using ImageJ. Indel mutation percentage was determined by the formula: $100 \times \left( 1 - \left( 1 - \frac{b + c}{a + b + c} \right)^{1/2} \right)$, where $a$ is the integrated intensity of the undigested PCR product, and $b$ and $c$ are the integrated intensities of each cleavage product.

**Southern Blot Analysis**

The external and internal probes were generated by PCR using the PCR DIG Probe Synthesis Kit (Roche). For the external probes we used HUES8 genomic DNA as the template. For internal probe generation we used the OCT4 2A-eGFP-PGK-Puro donor, OCT4-2A-mOrange donor and PDX1-2A-eGFP donor templates. For membrane hybridization, 5 µL of denatured DIG-labeled PCR product was added to 20 mL of hybridization buffer.

To identify correctly targeted hESC lines, genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). 5-10 µg of genomic DNAs was digested overnight with the appropriate restriction enzymes (OCT4 external and mOrange internal: BamHI, puromycin internal: EcoRI, PDX1 external: ApaLI, eGFP internal: ApaLI ) and then migrated in 1% agarose gels. The gel was denatured, neutralized, and transferred
overnight by capillarity on Hybond-N membranes (GE Healthcare) using 10x SSC transfer buffer. Hybridization with the external or the internal probe was carried out overnight at 65 °C. Probes were detected using an AP-conjugated DIG-Antibody (Roche) using CDP-Star (Roche) as a substrate for chemiluminescence as per manufacturer’s instructions.

**Immunofluorescence staining**

For immunostaining, cells were fixed with 4% paraformaldehyde for 10 minutes, washed once with phosphate buffered saline (PBS) and permeabilized in PBST (PBS + 0.1% Triton) for 15 minutes. Blocking was done for 5 minutes at RT with blocking solution (5% donkey serum in PBST). Primary and secondary antibodies were diluted in blocking solution. Primary antibodies were incubated at RT for 1 hr. The following primary antibodies were used at a 1:100 dilution: OCT4 (mouse monoclonal, Santa Cruz sc-5279); NANOG (rabbit polyclonal, CosmobioJapan REC-RCAB0004P-F); SOX2 (goat polyclonal, Santa Cruz sc-17320), RFP (rabbit polyclonal, Life Technologies R10367), GFP (rabbit polyclonal, Life Technologies A-6455). The PDX1 antibody was used at a 1:500 dilution (goat polyclonal, R&D AF2419). After primary antibody staining the cells were washed three times with PBST and then incubated with the appropriate Molecular Probes Alexa Fluor dye conjugated secondary antibodies (Life Technologies) for 1 hr.

**Reference:**

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., *et al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819-823.