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Supplemental Information

Using Gene Editing to Establish a Safeguard System for Pluripotent Stem-Cell-Based Therapies

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

Figure S1

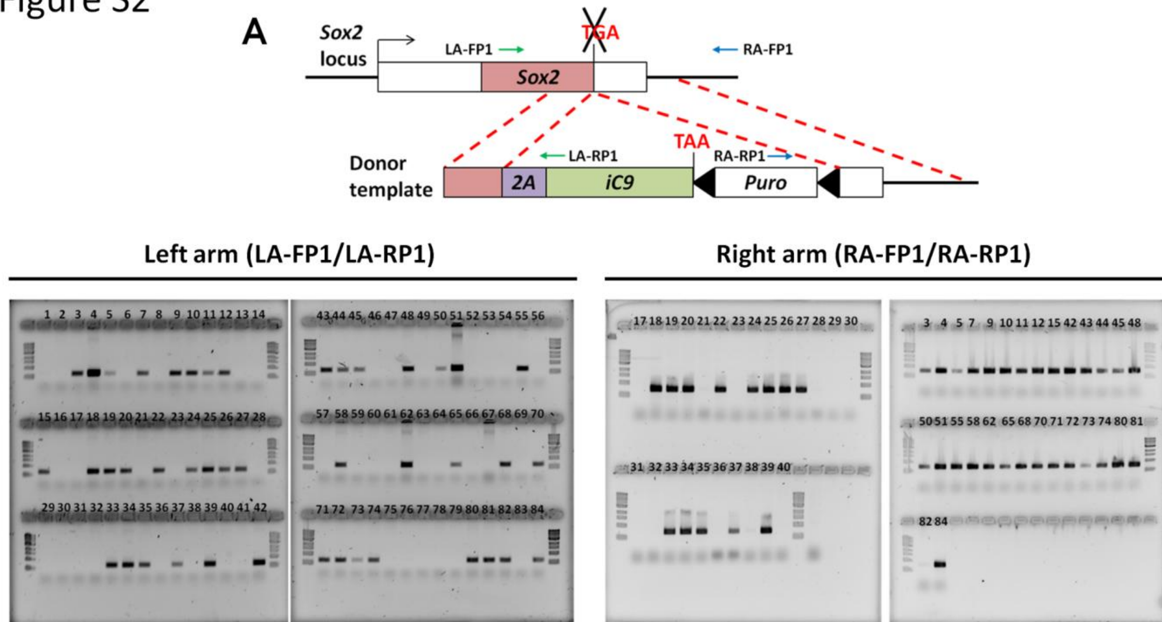
NANOG	Sequence	Identities
Chromosome 12 (7795053-7795135)	ATTCCTAAACTACTCCATGAACATGCAACCTGAAGACGTGTGAAGATGAGTGAAACTGATATTACTCAATTTCAGTCTGGACA	100%
Chromosome 12 (7898861-7898943)	…T…G…G…	96%
Chromosome 12 (7896574-7896656)	…T…G…	98%
Chromosome 15 (35084153-35084235)	…	100%
Chromosome 9 (100176014-100176093)	…T…A…G…TA…A…	93%

OCT4	Sequence	Identities
Chromosome 6 (31164561-31164643)	TGTCTCCGTCACCACTCTGGGCTCTCCCATGCATTCAAACTGAGGTGCCTGCCCTTCTAGGAATGGGGACAGGGGGAGGGGA	100%
Chromosome 12 (8133731-8133813)	…	100%
Chromosome 1 (155434221-155434302)	…C…	99%
Chromosome 8 (127416905-127416985)	…T…A…-…	96%
Chromosome 10 (68010166-68010246)	…T…T…G…AT…A…	93%
Chromosome 3 (128674629-128674706)	…T…TG…-…-…C…G…A…G…A…A…	86%

SOX2	Sequence	Identities
Chromosome 3 (181713272-181713354)	CGGCACGGCCATTAACGGCACACTGCCCCTCTCACACATGTGAGGGCCGGACAGCGAACTGGAGGGGGGAGAAATTTTCAAAG	100%
Chromosome 8 (124301767-124301849)	…C…A…G…A…G…	94%

Figure S1. Chromosomal locations and sequences of the genetic loci that show sequence similarity to the *NANOG*, *OCT4,* and *SOX2* genes, related to Figure 1. Genomic sequences spanning the stop codon of the *NANOG*, *OCT4,* and *SOX2* genes are shown. Stop codons of the three genes are boxed. Mismatched nucleotides in these genetic loci are marked by red and the percentage of sequence homology is on the right.

Figure S2



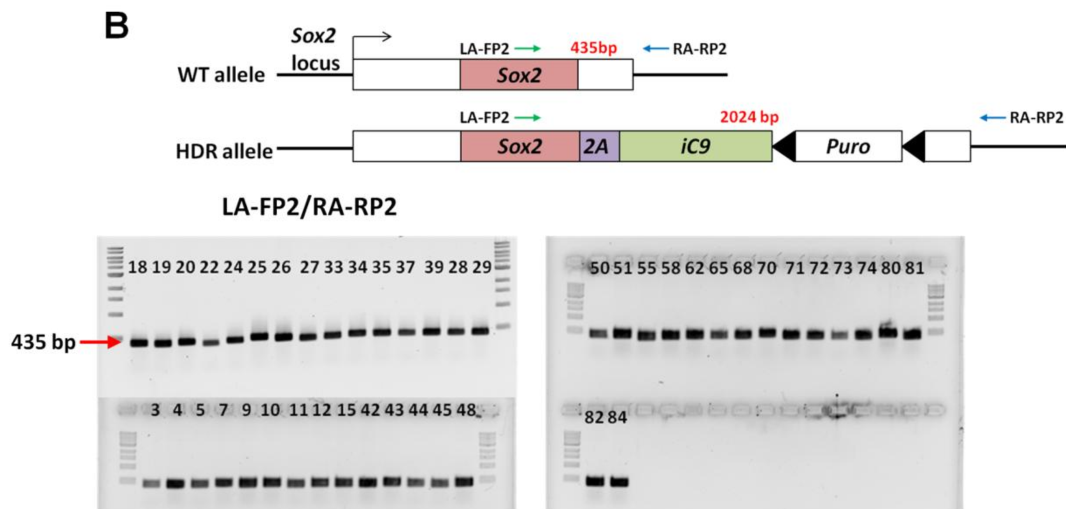


Figure S2. Establishment of the H1-iC9-pur clone, related to Figure 1. (A) Screening of H1 clones containing insertion of the *iC9* gene into the *SOX2* locus. Top: scheme for clone screening by genomic PCR. Two PCR primer pairs (green and blue arrows) and their sequences are listed in Table S2. Black arrowhead denotes the loxP site. Bottom: screening results from genomic PCR using the indicated primer pair. (B) Analysis of the positive clones identified in (A) for mono- and bi-allelic insertion of the *iC9* gene. Top: scheme for clone screening by genomic PCR. Primer pair used (green and blue arrows) and sequences are listed in Table S2. Bottom: screening results from genomic PCR using the indicated primer pair. Result indicates that all positive clones identified in (A) have a mono-allelic *iC9* gene insertion. (C) Sequence analysis of the two *SOX2* alleles near the stop codon of H1-iC9-pur cells.

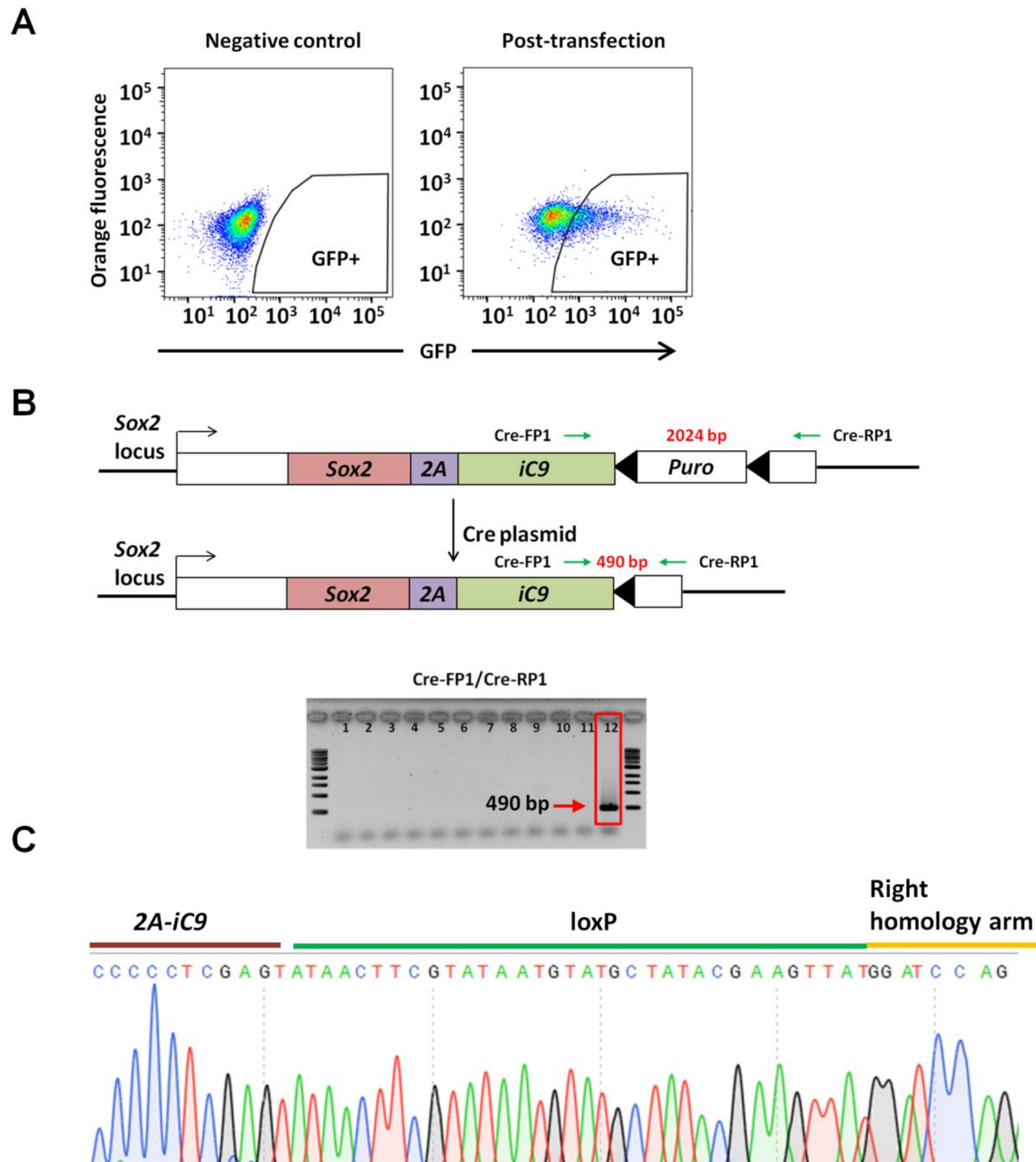


Figure S3. Isolation of the H1-iC9 clones, related to Figure 1. (A) Cell sorting of H1-iC9-pur cells co-transfected with pBS185 and pmaxGFP™. GFP+ cells were sorted 48 h after transfection and plated for clone isolation. (B) Isolation of the H1-iC9 clones. Top: scheme for genomic PCR screening to identify clones with the deletion of the puromycin-resistant gene. Positions of the PCR primers are indicated, and their sequences are listed in Table S2. Clones

without successful deletion of the puromycin-resistant gene are not expected to exhibit a PCR product due to the large fragment size. Bottom: A representative gel of genomic PCR result. The positive clone indicated by a red box had the expected deletion of the puromycin-resistant gene. (C) Sequence analysis of the allele containing the *iC9* gene in H1-iC9 cells confirmed deletion of the puromycin-resistant gene.

Figure S4

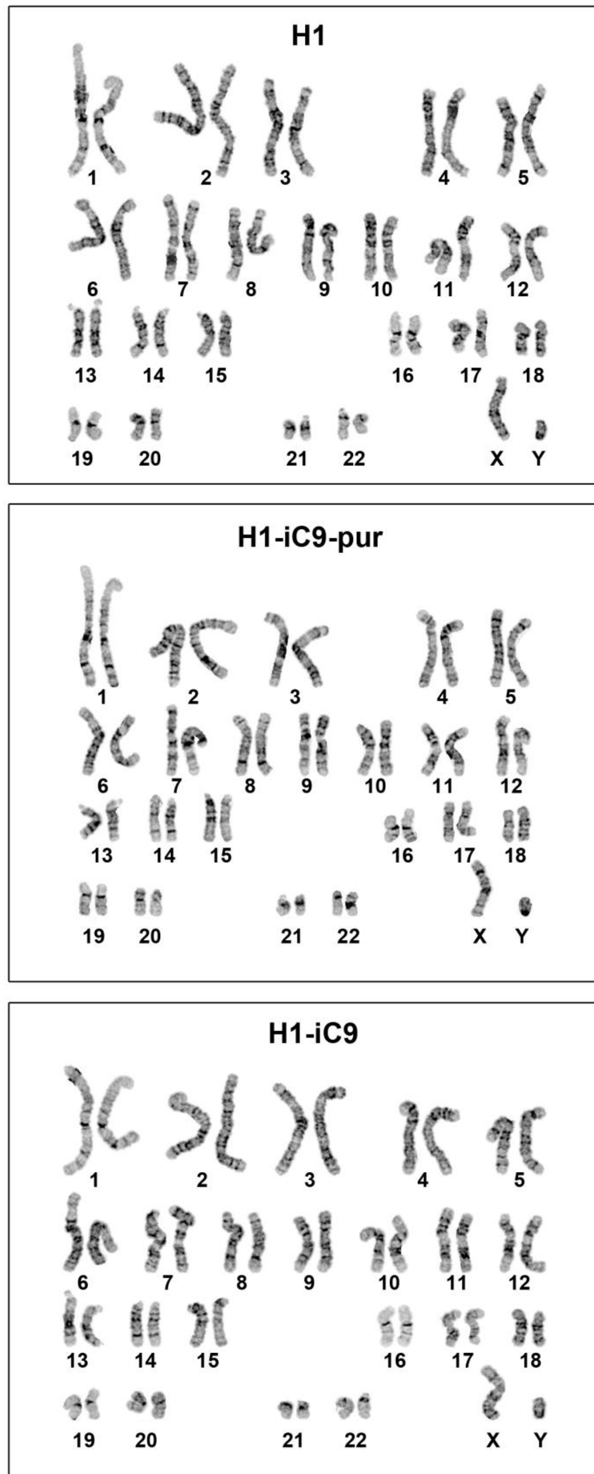
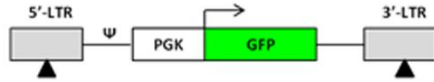


Figure S4. Karyotype analysis of H1, H1-iC9-pur and H1-iC9 cell lines, related to Figure 1.

Figure S5

A



B

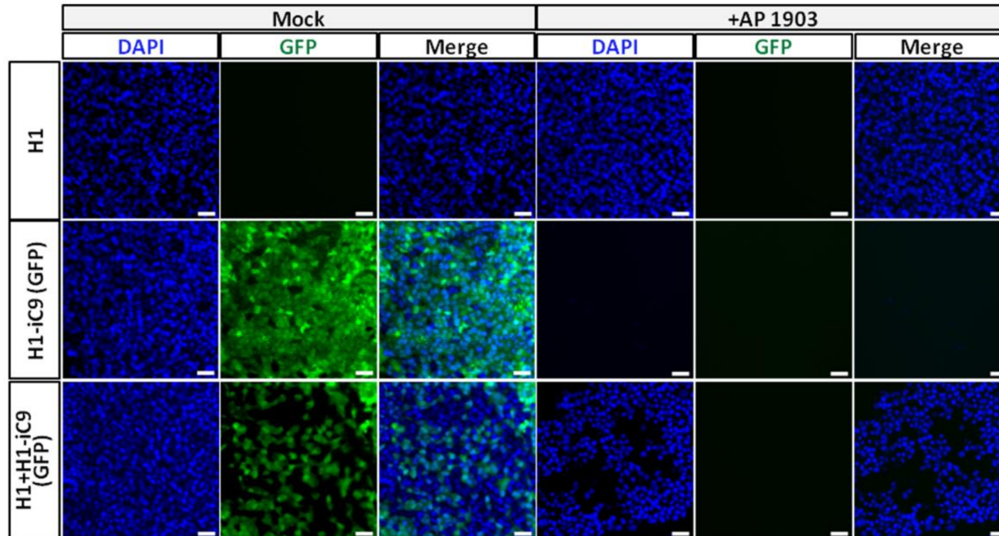


Figure S5. Selective eradication of H1-iC9 cells by AP1903 *in vitro,* related to Figure 2. (A) Marking of H1-iC9 cells with GFP. Structure of the lentiviral vector containing the *GFP* gene is shown. H1-iC9 cells were transduced with the GFP vector at a multiplicity of infection of 2, followed by cell sorting 48 h after transduction. GFP^+ cells were pooled and expanded. (B) H1 and H1-iC9(GFP) cells were either cultured alone or mixed in a 1:1 ratio. Each cell population was either mock treated or treated with 10 nM AP1903 overnight, and cell images were taken by fluorescence microscopy. The result indicates that AP1903 treatment selectively eradicates H1-iC9(GFP) cells in a mixed cell population containing both H1 and H1-iC9(GFP) cells. Scale bar = 50 μm.

Figure S6

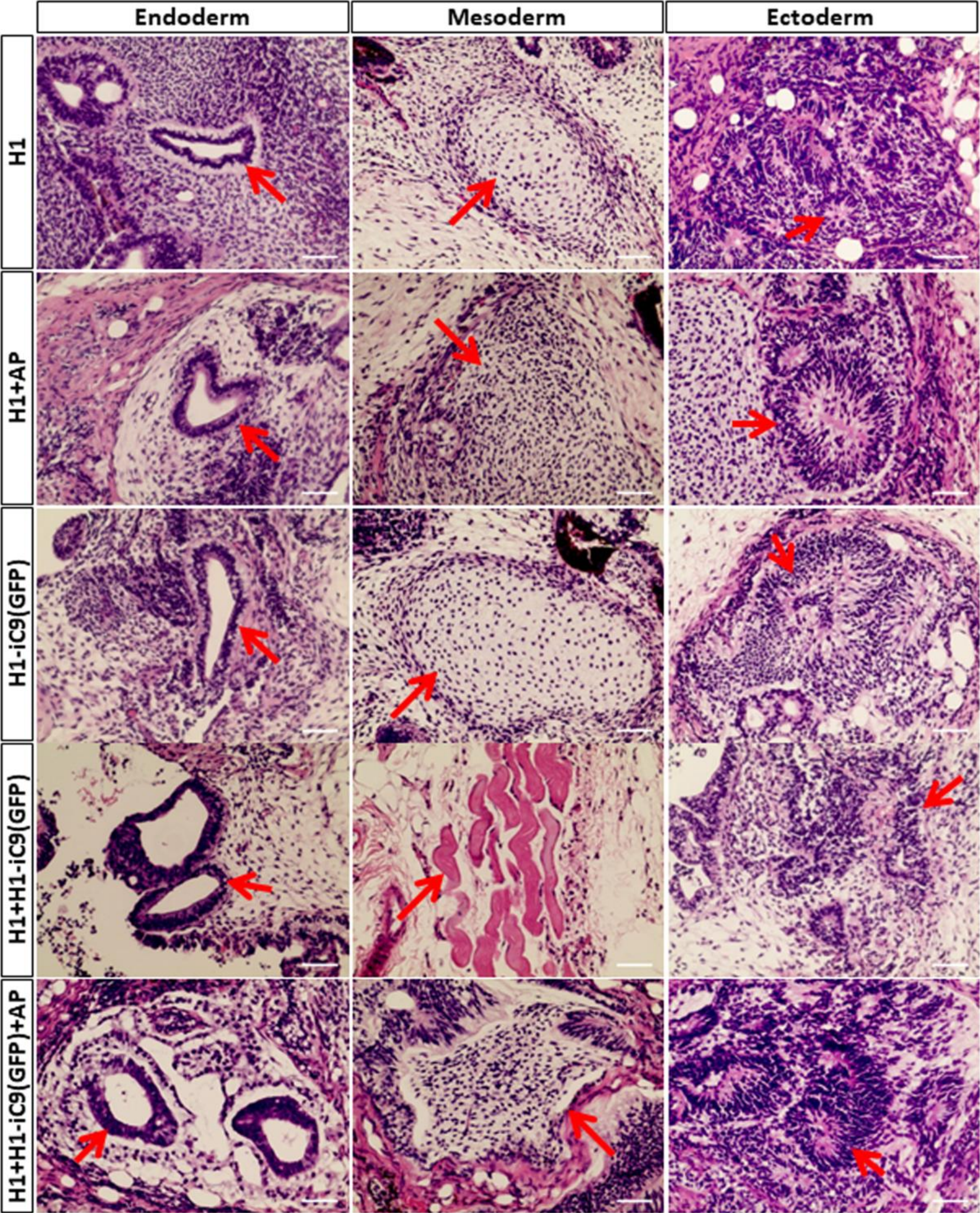


Figure S6. Teratoma formation in NSG mice with H1-iC9(GFP) cells, related to Figure 2.

Representative H&E staining of teratoma sections derived from indicated cells exhibits all three germ layers (arrows). Scale bar = 50 μm.

Figure S7

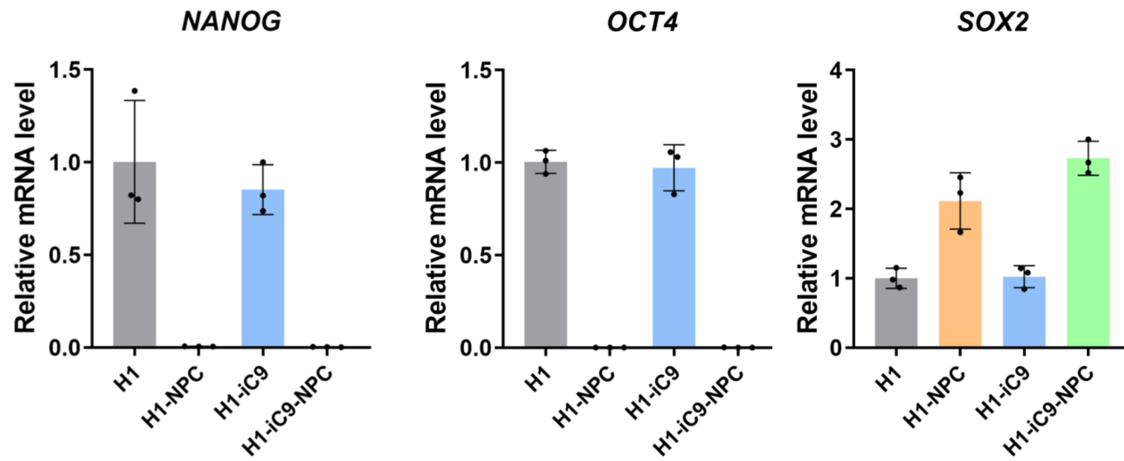


Figure S7. Relative expression of NANOG, OCT4 and SOX2 in undifferentiated cells and NPCs quantified by qRT-PCR, related to Figure 4. Graphs present the mRNA level normalized to the control group (H1) ± s.d. from three independent experiments.

Figure S8

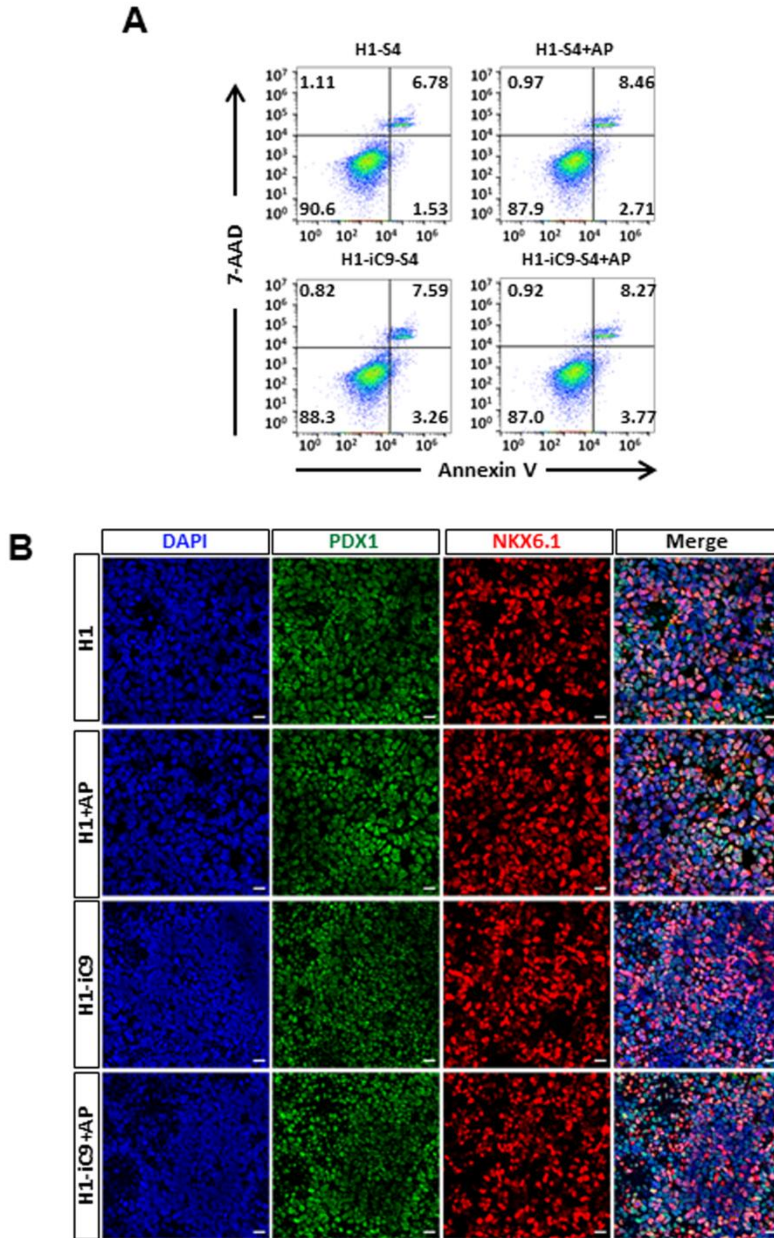


Figure S8. Viability of stage 4 pancreatic progenitor cells derived from H1 and H1-iC9 is not affected by AP1903 treatment, related to Figure 5. (A) Analysis of apoptotic cells in stage 4 pancreatic progenitor population derived from either H1 or H1-iC9 cells after overnight treatment with 10 nM AP1903. Result shown is representative of two independent experiments. (B) Representative immunofluorescence staining of $PDX1^{+}$ and $NKX6.1^{+}$ pancreatic progenitor

cells in the stage 4 population either mock treated or treated with 10 nM AP1903 overnight.

Scale bar = 20 μm.

Figure S9

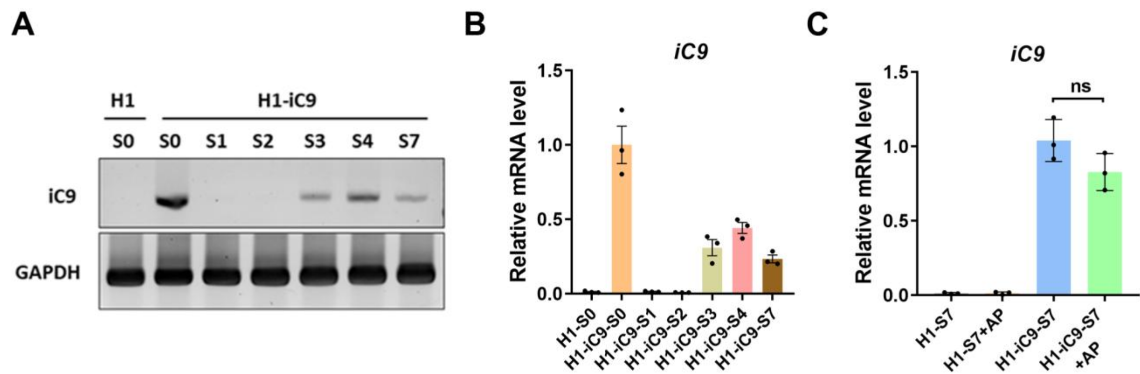


Figure S9. Transcription of transgene iC9 during beta-like cell differentiation, related to Figure 6. (A) Semi-quantitative RT-PCR analysis of the SOX2-iC9 fusion transcript in cells at the indicated differentiation stage. (B) Relative expression of the iC9 transgene in the indicated cells. The graph presents mean mRNA levels ± s.d. from three independent experiments normalized to the level in H1-iC9-S0 cells. (C) Relative expression of iC9 transgene in the S7 cells derived from H1 or H1-iC9 cells with and without AP1903 treatment, as measured by qRT-PCR (N=3). Data represents the mean ± s.d. ns: not statistically significant.

Figure S10

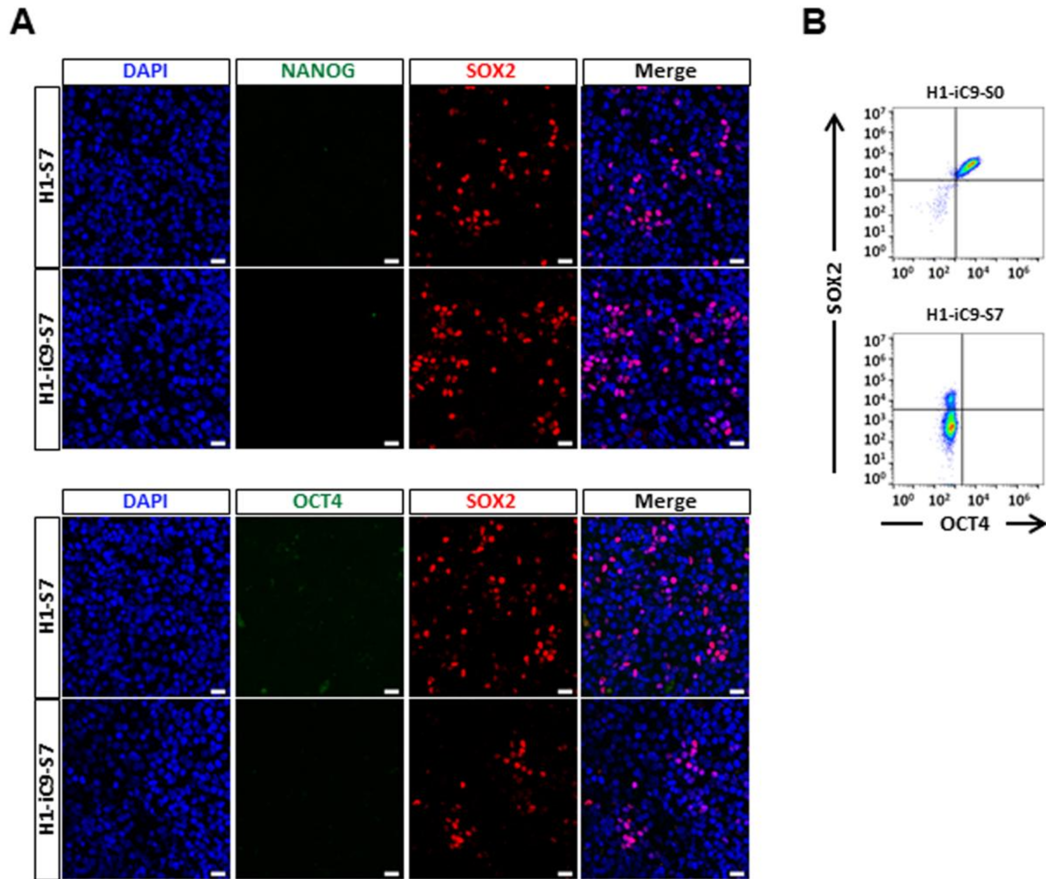


Figure S10. SOX2⁺ cells in the S7 population are not undifferentiated stem cells, related to Figure 6. (A) Stage 7 cell populations derived from H1 and H1-iC9 differentiation were co-stained with antibodies against NANOG, OCT4, and SOX2 and detected by fluorescence microscopy. Scale bar = 20 μm. (B) Flow cytometry analysis of $SOX2^+/OCT4^+$ cells in the S0 and S7 populations derived from H1-iC9 cells. Result shown is a representative of two independent experiments.

Figure S11

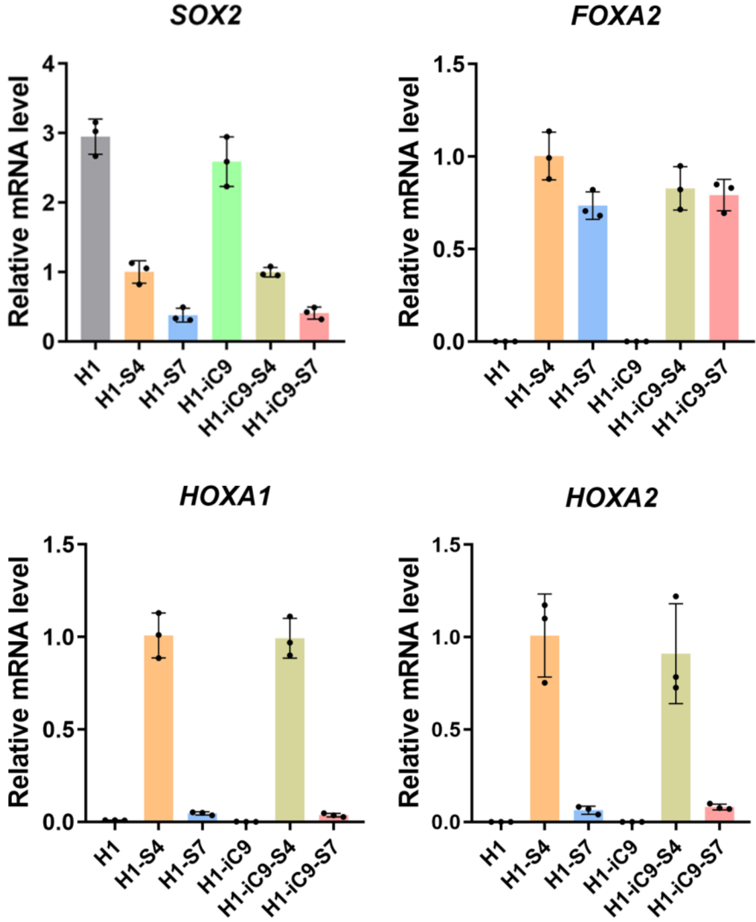


Figure S11. Relative mRNA level of SOX2, FOXA2, HOXA1 and HOXA2 in the indicated cells, related to Figure 6. Data are presented as the fold change relative to H1-S4 cells (N=3 biological replicates).

SUPPLEMENTAL TABLES

Table S1. Sequencing analysis of predicted off-target sites, related to Figure 1.

Genomic location	CRISPR Target Sequence	Sequencing results
chr3:181713280 (on target)	CCATTAACGGCACACTGCCCCTC	
chr8:124301775	CCATTAACGaCACACTGCCCCTC	WT
chr12:116042336	GAGGGGCAGgaTGCCtTTAATGG	WT
chr14:74269072	GAGGGGCAGaGTaCCGTgAAAGG	WT
chr19:44685040	GAGGGGCAGgGTGCtGTgAAAGG	WT
chr2:127627389	GAGGGGCAGTGTGCCGTcccGGG	WT
chr3:181713318 (on target)	CCGGACAGCGAACTGGAGGGGGG	
chr3:136862390	CCCCCCTtCAGTcCGCTGTCCGt	WT
chr10:49752274	CCGGgCAGCaAAgTGGAGGGAGG	WT
chr17:30210703	CaGGACAGtGAACaGGAGGGAGG	WT
chr5:443399	CCGGACcGgGAcCTGGAGGGCGG	WT
chr6:31781643	CCACCCTCCAGTcCcCTGTCCcG	WT

Table S2. Primers used in this study, related to Figure 1-3 and Figure 6.

Primers for plasmid construction

Donor plasmid	PCR Fragments	Primer set
2A-iC9-Puro	Left arm	FP: CCATCGATGAACCAGCGCATGGACAGTTAC RP: GCTCTAGACATGTGTGAGAGGGGCAGTGTGCCGTTAATAGCCGTGCCGG
	Right arm	FP: GCTCTAGAAAGCTTGGATCCAGAAATTTTCAAAGAAAAACGAGGGAAAT RP: AAGGAAAAAAGCGGCCGCGCAAGAAGCCTCTCCTTGAAAAATATT
	2A	FP: AAGGAAAAAAGCGGCCGCTCTAGACTCGAGGGCAGCGGCGCCACGAACTTC RP: AGATAGTCTCCACCTGCACTCCAGGACCGGGGTTTTCTT
	FKBP	FP: ATAAGAATGCGGCCGCAATGGGAGTGCAGGTGGAGACTATCT RP: GTCGACTCCGGATCCACCGCCAGATTCCAGTTTTAGAA

	2A-FKBP	FP: AAGGAAAAAAGCGGCCGCTCTAGACTCGAGGGCAGCGGCGCCACGAACTTC RP: GTCGACTCCGGATCCACCGCCAGATTCCAGTTTTAGAA

Primers for Surveyor assay

Target Gene	Primer set	Expected PCR product (bp)	Expected cleaved products (bp)
SOX2	FP: TGCAGGACCAGCTGGGCTAC RP: CGTGAGTGTGGATGGGATTGGT	581	405, 176

Primers for clone screening

Target Gene	PCR region	Primer set
SOX2	Left arm junction	LA-FP1: GCGGCAATAGCATGGCGA LA-RP1: AGATAGTCTCCACCTGCACTCCCATAGGACCGGGGTTTTCTT
	Right arm junction	RA-FP1: GTGTCTCTCACTCGGAAGGACAT RA-RP1: TCTTCTTTTCACGTTTGCAACTGTC
	Monoallelic and biallelic gene insertion	LA-FP2: CCATGGGTTCGGTGGTCAAGT RA-RP2: CGTGAGTGTGGATGGGATTGGT
	Puromycin-resistant gene deletion	Cre-FP1: TCCTGGTACGTTGAGACCCTGG Cre-RP1: CGTGAGTGTGGATGGGATTGGT

Primers for RT-PCR

Target Gene	Primer set	Expected PCR product size (bp)
SOX2	FP: GGGAAATGGGAGGGGTGCAAAAGAGG RP: TTGCGTGAGTGTGGATGGGATTGGTG	151
SOX2 fusion transcript	FP: CCATGGGTTCGGTGGTCAAGT RP: CCACATCGAAGACGAGAGTGG	621
OCT4	FP: TCGAGAACCGAGTGAGAGG RP: GAACCACACTCGGACCACA	125
NANOG	FP: ATGCCTCACACGGAGACTGT RP: AAGTGGGTTGTTTGCCTTTG	103
iC9	FP: CAGGAGACGTGGAAGAAAACCCC RP: TAGTGCACCACGCAGGTCTGG	109
FOXA2	FP: GGAGCAGCTACTATGCAGAGC RP: CGTGTTCATGCCGTTCATCC	83
PAX9	FP: GGAGGAGTGTTCGTGAACGG RP: CGGCTGATGTCACACGGTC	98
HOXA1	FP: TCCTGGAATACCCCATACTTAGC RP: GCACGACTGGAAAGTTGTAATCC	95

HOXA2	FP: ACCCCGAAGGGTGGAGATT RP: CGGAGTCCTCAAGGCTTTTACAT	152
GAPDH	FP: TGCACCACCAACTGCTTAGC RP: GGCATGGACTGTGGTCATGAG	87

Table S3. Antibodies for flow cytometry and immunofluorescence staining, related to Figure 2-6.

Antibodies for flow cytometry

Conjugated antibody	Source	Dilution
FITC Mouse anti-CD45	BioLegend	1:40
Alexa Fluor 647 Mouse anti-SOX2	BD Biosciences	1:40
PE Mouse anti-Oct3/4	BD Biosciences	1:10
Alexa Fluor 647 Mouse anti-NKX6.1	BD Biosciences	1:40
PE Rabbit anti-Insulin	Cell Signaling Technology	1:100
Alexa Fluor 647 Mouse IgG1, κ isotype control	BD Biosciences	1:40
PE Mouse IgG1, κ Isotype Control	BD Biosciences	1:10
PE Rabbit IgG1 XP isotype control	Cell Signaling Technology	1:100

Antibodies for immunofluorescence staining

Primary antibodies			
Antigen	Species	Source	Dilution
Nestin	Rabbit	EMD Millipore	1:500
NANOG	Mouse	BD Biosciences	1:500
OCT4	Rabbit	Stemgent	1:500
SOX2	Rabbit	Novus Biology	1:250
SOX2	Goat	R&D System	1:100
TUJ1	Rabbit	Covance	1:1000
PDX1	Rabbit	Abcam	1:500
NKX6.1	Mouse	BCBC #2023	1:500
MAFA	Rabbit	Abcam	1:500
Insulin	Guinea Pig	Dakocytomation	1:1000
GFP	Chicken	Abcam	1:2000
HA	Rabbit	Cell Signaling Technology	1:800
FOXA2	Goat	R&D System	1:25
Secondary antibodies			
Antigen	Conjugation	Source	Dilution
Rabbit/Mouse/Chicken	Alexa Fluor 488	Jackson ImmunoResearch	1:1000

Goat/Rabbit	Cy3	Jackson ImmunoResearch	1:2000
Guinea Pig/Sheep	Cy5	Jackson ImmunoResearch	1:500

Table S4. Protocol for H1 differentiation into stage 7 beta-like cells, related to Figure 5.

Stage	Day	Basic medium	Factors
Stage 1	Day 1	MCDB 131 1x Glutamax 10 mM Glucose 1.5 g/L Sodium bicarbonate 0.5% BSA	100 ng/mL GDF8 (PeproTech) 3 μM CHIR99021 (SellechChem)
	Day 2	MCDB 131 1x Glutamax 10 mM Glucose 1.5 g/L Sodium bicarbonate 0.5% BSA	100 ng/mL GDF8 0.3 μM CHIR99021
	Day3	MCDB 131 1x Glutamax 10 mM Glucose 1.5 g/L Sodium bicarbonate 0.5% BSA	100 ng/mL GDF8
Stage 2	Day4-5	MCDB 131 1x Glutamax 10 mM Glucose 1.5 g/L Sodium bicarbonate 0.5% BSA	50 ng/mL FGF7 (PeproTech) 0.25 mM ascorbic acid (Sigma)
Stage 3	Day 6-7	MCDB 131 1x Glutamax 10 mM Glucose 2.5 g/L Sodium bicarbonate 2% BSA	50 ng/mL FGF7 0.25 mM ascorbic acid 0.25 μM SANT-1 (Sigma) 1 μM retinoic acid (Sigma) 100 nM LDN193189 (Stemgent) 1:200 ITS-X (Life technologies) 200 nM TPB (Millipore)
Stage 4	Day 8-10	MCDB 131 1x Glutamax 10 mM Glucose 2.5 g/L Sodium bicarbonate 2% BSA	2 ng/mL FGF7 0.25 mM ascorbic acid 0.25 μM SANT-1 0.1 μM retinoic acid 200 nM LDN193189 1:200 ITS-X 100 nM TPB
Stage 5	Day 11-13	MCDB 131 1x Glutamax 20 mM Glucose 1.5 g/L Sodium bicarbonate 2% BSA	0.25 μM SANT-1 0.05 μM retinoic acid 100 nM LDN193189 1:200 ITS-X 1 μM T3 (Sigma) 10 μM ALK5i (Enzo Life Sciences) 10 μM zinc sulfate (Sigma) 10 μg/mL heparin (Sigma)
Stage 6	Day 14-20	MCDB 131 1x Glutamax 20 mM Glucose 1.5 g/L Sodium bicarbonate	100 nM LDN193189 1:200 ITS-X 1 μM T3 10 μM ALK5i

		2% BSA	10 μM zinc sulfate 100 nM Gs inh XX (EMD Millipore) 10 μg/mL heparin
Stage 7	Day 21-27	MCDB 131 1x Glutamax 20 mM Glucose 1.5 g/L Sodium bicarbonate 2% BSA	1:200 ITS-X 1 μM T3 10 μM ALK5i 10 μM zinc sulfate 1 mM N-acetyl cysteine (Sigma) 10 μM Trolox (EMD Millipore) 2 μM R428 (SelleckChem) 10 μg/mL heparin

TRANSPARENT METHODS

Plasmid construction

Cas9(phCas9) (Mali et al., 2013) and Cas9 D10A (phCas9-D10A) (Mali et al., 2013) expression plasmids were purchased from Addgene (Cambridge, MA). The CRISPR plasmid, pU6-CRISPR, containing the U6 promoter and the gene for the single-guide RNA (sgRNA) was generated as previous described (Wang et al., 2015). To generate the donor plasmid for in-frame insertion of the *iC9* gene into the *SOX2* locus, left and right homology arms sequences (~0.5 kb of each) spanning the stop codon of the *SOX2* gene were PCR amplified from the genomic DNA of H1 cells and cloned into pBluescript SK(-) to generate pBS-SOX2-HA. The 1.3 kb *2A-iC9* gene was constructed by in-frame fusion of the PCR amplified *iC9* gene in pMSCV-F-del Casp9. IRES.GFP (Addgene) (Straathof et al., 2005) with a 57-bp synthetic fragment encoding the 2A peptide. The *2A-iC9* cassette was then cloned into pBluescript SK(-) to generate pBS-2A-iC9. To construct the donor plasmid, pBS-SOX2-HA was linearized by Xba1 and BamH1 double digestion. A 1.3 kb fragment containing the *2A-iC9* gene was isolated from pBS-2A-iC9 by Xba1 and Kpn1 double digestion. A 1.6 kb fragment containing the pgk-Puro fragment flanked by loxP recognition sites was isolated from ppgk-Puro by Kpn1 and BamH1 digestion. The three fragments were ligated to generate pSOX2-iC9-donor. All primers used for plasmid construction are listed in Table S2.

DNA transfection

HEK293T cells were cultured in DMEM (Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were passaged twice a week at 37°C under 5% CO_2. For the Surveyor assay, HEK293T cells cultured in 48-well plates were transfected at 40% confluency using Lipofectamine 3000 (Life Technologies) together with 67 ng pU6-CRISPR and 200 ng of Cas9 or Cas9 D10A expression plasmid. Forty-eight hours after transfection, genomic DNA was isolated with Epicentre QuickExtract solution (Epicentre Biotechnologies, Madison, WI) and used for the Surveyor assay. Human ES cell line H1 (WA01) was purchased from WiCell Research Institute (Madison, WI). The cells were cultured in mTeSR1 medium (StemCell Technologies, Vancouver, BC) on plates coated with Matrigel (BD Bioscience, San Jose, CA). Cells were passaged with ReLeSR (STEMCELL Technologies) every 4-6 days with 1:6~1:12 dilutions. For DNA transfection, H1 cells pretreated overnight with Rho kinase (ROCK)-inhibitor Y-27632 (Stemgent, Beltsville, MD) were dissociated into single cells using Accutase (STEMCELL Technologies). Nucleofection was carried out using 4D Amaxa P3 Primary Cell 4-D Nucleofector system (Lonza). Approximately $8X10^5$ cells were nucleofected using program CA-137. To insert the *iC9* gene into the *SOX2* locus in H1 cells, 1.5 µg pSOX2-iC9-donor was nucleofected with 0.65 µg pU6-sgRNA1, 0.65 µg pU6-sgRNA2, and 2 µg pCas9 D10A. Two days after nucleofection, single cells were prepared and plated in mTeSR1 medium containing 0.5 µg/mL puromycin. Puromycin-resistant colonies were picked after two weeks of selection and genotyped with genomic PCR using primers listed in Table S2. To delete the pgk-Puro cassette, 4 µg pBS185 (Sauer, 1993) and 1 µg pmax-GFP™ (Lonza) were introduced into cells by nucleofection. FACS sorted GFP^+ cells were plated and individual colonies picked after two weeks. Genomic PCR was performed to verify the deletion of the puromycin cassette. PCR primers used for genotyping the clones are listed in Table S2.

Surveyor assay

To carry out the Surveyor assay, the genomic DNA spanning the CRISPR cleavage site was PCR amplified with Hotstar Taq (Qiagen, Germantown, MD), and the PCR product was subjected to denaturation and reannealing with the following conditions: 95°C for 10 min, 95°C to 85°C ramping at –2°C/s, 85°C to 25°C ramping at – 0.25°C/s, and 25°C for 1 min. PCR primers for DNA amplification are listed in Table S2. Three microliter of re-annealed DNA in a 12 µL reaction was mixed with 1 µL of Surveyor Nuclease S (Integrated DNA Technologies, Skokie, IL), 1 µL of Surveyor Enhancer S, and 1.2 µL of 0.15 M $MgCl_2$ and incubated at 42°C for 1 h. The digested product was analyzed on a 2% agarose gel and imaged with a ChemiDoc imaging System (Bio-Rad Laboratories, Hercules, CA). The percentage of indel formation was calculated by the following formula: % indel formation = $100 \times [1 - (1 - \text{fraction cleaved})^{1/2}]$; fraction cleaved = 100 × sum of the cleavage product peak / cleavage product + parent peak.

Lentiviral production and transduction

To generate the lentiviral vector HIV7/PGK-GFP, approximately 2×10^6 HEK293T cells were cotransfected with 12 µg pHIV7/PGK-GFP, 12 µg pCgp, 4 µg pRev-2, and 2 µg pCMV-G (Lo et al., 2007) using calcium phosphate coprecipitation. Infectious virus was harvested 48 h later and tittered on HT1080 cells with different dilutions in the presence of 4 µg/mL polybrene. FACS analysis was carried out 48 h after transduction to determine the fraction of GFP^+ cells.

Cytogenetic Analysis

ES cells at ~80% confluence in mTesR culture medium were processed for preparation of metaphase chromosome spreads according to standard cytogenetic protocols (Barch and Association of Cytogenetic Technologists., 1991). Following DAPI staining, metaphase chromosome spreads were observed and counted under a fluorescence microscope. Twenty metaphases were analyzed for each cell line.

Cell viability assay

H1, H1-iC9-pur and H1-iC9 cells were seeded in 96-well plate at a density of 10^4 cells /well in the absence or in the presence of varying concentrations of AP1903 overnight. Cell viability was determined by MTS assay using a CellTiter 96® AQueous One Solution kit (Promega, Madison, MI) according to the manufacturer's instructions.

Apoptosis assay

Apoptosis assay was performed after overnight incubation of cells with 10 nM AP1903. To block Caspase activity, the pan Caspase inhibitor qVD-OPh (20 μmol/l, ApexBio Technology, Boston, MA) was added together with AP1903. The percentage of apoptotic cells after overnight incubation was measured by staining the cells with Annexin V-PE and 7-amino actinomycin D (7-AAD) following the manufacturer's instruction (BD Pharmingen, San Jose, CA) and analyzed by flow cytometry. Annexin V^+/7-AAD^- and Annexin V^+/7-AAD^+ cells represent early and late apoptotic cells, respectively.

Teratoma formation

The stable H1-iC9(GFP) line was established by the transduction of H1-iC9 cells with HIV7/C-GFP at a multiplicity of infection of 2. GFP^+ cells were isolated and pooled by cell sorting 48 h after transduction. To induce teratoma, cells were dissociated with Accutase and resuspended in mTeSR1 medium containing 10 μM Y-27632. Approximately $2X10^6$ cells in 50 μL medium were mixed with 50 μL cold Matrigel (BD Pharmingen) and injected subcutaneously into the lateral flank of 8-12 week old NOD scid gamma (NSG) mice. Teratomas were harvested 4-6 weeks after cell injection, fixed in 10% neutralized formalin for 24 h, embedded in paraffin, and

sectioned for hematoxylin-eosin (H&E) staining. Animal studies were carried out in accordance with protocols approved by the City of Hope Institutional Animal Care and Use Committee.

Semi-quantitative RT-PCR and real-time RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. The purity and concentration of RNA were determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (1 μg) was reverse transcribed into cDNA using SuperScript VILO MasterMix (Life Technologies). For semi-quantitative PCR, 5 μL diluted cDNA (1:25) was amplified using HotStar Taq polymerase. The products were separated on a 2% agarose gel and imaged with ChemiDoc imaging System. Real-time RT-PCR was carried out in the CFX96 Touch™ Real-time PCR Detection System (Bio-Rad) using SYBR Green Supermix (Bio-Rad). Primers used for RT-PCR are listed in Table S2.

Flow cytometry analysis

To detect $CD45^+$ hematopoietic cells, both H1- and H1-iC9-derived hematopoietic cell populations were suspended in flow cytometry staining buffer (Thermo Fisher Scientific, Irwindale, CA). FITC-conjugated anti-human CD45 antibody (Biolegend, San Diego, CA) at a 1:40 dilution was added and the mixture incubated for 30 min at room temperature. For the staining of intracellular antigens, cells in suspension were fixed and permeabilized using Foxp3 transcription factor staining kit (Thermo Fisher Scientific). After washing with permeabilization buffer, cells were stained with directly conjugated antibodies. Antibodies and dilutions used for FACS are listed in Table S3. The data for FACS was collected using an Accuri C6 flow Cytometer (BD Biosciences) and analyzed with the FlowJo software.

Immunofluorescence staining

For cell immunofluorescence staining, monolayer cultures were fixed in 4% paraformaldehyde for 15 min at room temperature and washed with PBS three times, followed by incubation for 1 h in blocking solution containing 5% BSA and 0.3% Triton X-100 in PBS. Primary and secondary antibodies were diluted with 1% BSA and 0.15% Triton X-100 in PBS. Cells were incubated with primary antibodies at 4°C overnight. After three washes with PBS, cells were incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature. After three washes with PBS, the cell-containing slide was mounted with mounting reagent and stained with DAPI (Vector Laboratories, Burlingame, CA). Images were captured using a Zeiss Axio Imager microscope (Carl Zeiss, San Diego, CA). For the staining of H1- and H1-iC9-derived beta-like cells, cell clusters in S7 were removed from air-liquid interface, washed with PBS, and fixed in 4% paraformaldehyde solution at 4°C for 1 h. After three washed with PBS, cell clusters were incubated in 30% sucrose at 4°C overnight, embedded in OCT compound (Sakura, Torrance, CA), and frozen in dry ice. Sections (5 µm thickness) were prepared using a cryostat (Leica, Allendale, NJ). Slides were washed with PBS to remove OCT compound followed by incubation with blocking solution and stained with primary and secondary antibodies as described above. For paraffin-embedded teratoma staining, sections were deparaffinized in Clearify™ (American MasterTech, Lodi, CA), rehydrated with a gradient of ethanol, and underwent antigen retrieval in citrate buffer (PH 6.0) before blocking and antibody staining. Primary and secondary antibodies are listed in Table S3.

Western blot

Harvested cells were lysed using RIPA buffer (Thermo Fisher Scientific) containing HALT™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). The cell lysates were mixed with 4xNuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) and loaded onto a NuPAGE 4–12%

Bis-Tris gel (Invitrogen) for protein separation. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and blocked for 1 h with 5% BSA and 0.05% Tween in PBS followed by the incubation with a BCL-2 (50E3) rabbit antibody (1:1000, Cell Signaling Technology, Danvers, MA) or a mouse GAPDH antibody (1:2000, GeneTex, Irvine, CA) diluted with blocking solution at 4^0C overnight. Membranes were washed with PBS and incubated with HRP-conjugated secondary antibodies (Thermo Fisher Scientific) at room temperature for 1 h and developed using Pierce ECL substrate (Thermo Fisher Scientific).

Hematopoietic cell differentiation

Undifferentiated human H1 cells or H1-iC9 cells were induced to differentiate into $CD45^+$ cells as previously described (Senju et al., 2011). Briefly, undifferentiated cells were transferred onto OP9 feeder layers and cultured with α-Minimal Essential Medium (Hyclone) supplemented with 20% fetal calf serum. After 18 days, cells were detached with dissociation solution containing 0.25% trypsin, 0.1% collagenase type IV (Sigma), and 0.1% DNase I (Sigma) in PBS. Cells were passed through 100 μm cell strainers (Corning, Corning, NY) to yield a single-cell suspension, followed by culturing the cell suspension in α- Minimal Essential Medium containing 100 ng/mL GM-CSF and 50 ng/mL M-CSF (PeproTech, Rocky Hill, NJ) for 14 days.

Neuronal cell differentiation

Approximately 3×10^6 Accutase-dissociated single cells were suspended in 1ml neural induction medium consisting of STEMdiff™ Neural Induction Medium and SMADi Neural Induction Supplement (STEMCELL Technologies). Embryoid bodies (EBs) were harvested with 37 μm Reversible Strainers 5 days later followed by seeding into plates coated with Poly-L-ornithine (PLO) and laminin (Sigma) in STEMdiff™ Dopaminergic Neuron Differentiation Medium (STEMCELL Technologies). Neuronal rosettes that emerged were selectively removed with STEMdiff™ Neural Rosette Selector Reagent (STEMCELL Technologies) after 6 days in

culture. Rosettes were then replated into a new PLO/laminin-coated well and expanded for additional 7 days in STEMdiff™ Dopaminergic Neuron Differentiation Medium to generate neuronal precursors. Neuron precursors were frozen in culture medium supplemented with 10% DMSO. To generate neurons, frozen neuronal precursors were thawed and expanded for additional 7 days. The cells were then dissociated with Accutase and plated into PLO/laminin-coated 24-well plates at a density of 4~6 x 10^5/cm^2 in STEMdiff™ Dopaminergic Neuron Maturation Medium 1 (STEMCELL Technologies) for 2 days. AP1903 was added on the third day after the initiation of the maturation process. SOX2 expression was then examined 2 days after AP1903 treatment.

Beta-like cell differentiation

Human beta-like cells were differentiated from H1 and H1-iC9 cells using a seven-stage differentiation protocol reported by Rezania *et al*. (Rezania et al., 2014). Before differentiation, approximately 1.3-1.5 x 10^5 cells/cm^2 undifferentiated cells were seeded into Matrigel-coated plates in mTeSR medium supplemented with 10μM Y27632. After 48 h, cell differentiation was initiated by replacing the culture media with MCDB 131 media (Corning) supplemented with Glutamax (Life Technologies), fatty acid-free BSA (Proliant Biologicals, Boone, IA), and a cocktail of molecules described in Table S4 with fresh media change every day. Cells were cultured in monolayer during the first four stages of differentiation to generate pancreatic progenitor cells in stage 4. The stage 4 cells were dissociated with TrypLE Express Enzyme (Life Technologies) and resuspended in stage 5 media at a density of 5 x 10^7/mL. Approximately 10 μL cell drops containing 5 x 10^5 cells were spotted onto a filter insert (Corning) floating in 1.5 ml stage 5 differentiation media. Cells cultured in this air-liquid interface were allowed to differentiate into stage 7 with media change every day. Cell clusters were removed from the insert for analysis between 28~35 days after the initiation of differentiation.

Statistical analysis

Results are presented as mean ± standard deviation (s.d.). Statistical significance between groups was calculated using one-way ANOVA followed with Turkey's correction. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns: P value not significant. Statistic calculations were conducted using GraphPad Prism 7 software (GraphPad Software, San Diego, CA). The number of independent experiments was indicated in figure legends.

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