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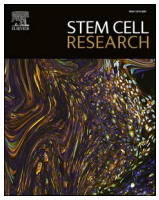

Lab Resource: Genetically-Modified Single Cell Line

Generation of SST-P2A-mCherry reporter human embryonic stem cell line using the CRISPR/Cas9 system (WAe001-A-2C)

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A B S T R A C T

Somatostatin (SST)-producing pancreatic delta-cells play an important role in maintaining the balance of insulin and glucagon secretion within the islets. This study aimed to generate a human embryonic stem cell (hESC) line with a SST-P2A-mCherry reporter using CRISPR/Cas9 system. The SST-P2A-mCherry reporter cell line was shown to maintain typical pluripotent characteristics and able to be induced into SST-producing pancreatic delta-cells. The generation of the cell line would provide useful platform for the characterization of stem cell-derived delta-cells, discovery of delta-cell surface markers and investigation of paracrine mechanisms, which will ultimately promote the drug discovery and cell therapy of diabetes mellitus.

Resource Table:

Unique stem cell line identifier	WAe001-A-2C
Alternative name(s) of stem cell line	SST-P2A-mCherry
Institution	Guangzhou Laboratory, Guangdong, China
Contact information of the reported cell line distributor	Huisheng Liu, liu_huisheng@gzlab.ac.cn
Type of cell line	Embryonic stem cell
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Sex: Male
Cell Source	H1 human embryonic stem cell
Method of reprogramming	N/A
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
The cell culture system used	mTeSR
Type of the Genetic Modification	Reporter knock-in
Associated disease	Diabetes
Gene/locus modified in the reported transgenic line	*SST*/3q27.3

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Method of modification / user-customisable nucleases (UCN) used, the resource used for design optimisation	CRISPR/Cas
User-customisable nuclease (UCN) delivery method	Plasmid transfection
All double-stranded DNA genetic material molecules introduced into the cells	CRISPR/Cas9 plasmid, sgRNA-plasmid, HDR donor vector
Evidence of the absence of random integration of any plasmids or DS DNA introduced into the cells.	N/A
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele, PCR (heterozygous allele)
Homozygous allele status validation	PCR and Sanger sequencing
Method of the off-target nuclease activity prediction and surveillance	N/A
Descriptive name of the transgene	P2A-mCherry-NLS-PGK-puro-PGK-polyA
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	Puromycin
Inducible/constitutive expression system details	N/A

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(*continued*)

Date archived/stock creation date	9th December 2022
Cell line repository/bank	https://hpscreg.eu/cell-line/WAe001-A-2C
Ethical/GMO work approvals	This study was approved by the ethics committee of the Guangzhou laboratory
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

1. Resource utility

The SST-P2A-mCherry hESC reporter line would provide a convenient tool to purify the hESC-derived SST-producing cells, thereby establishing useful platforms for the potential delta-cell surface marker identification, *in vivo* and *in vitro* functionality assessment of delta-cells, drug discovery as well as further applications in cell therapy of diabetes (see Table 1).

2. Resource details

Pancreatic islets are complex micro-organs consisting of pancreatic exocrine and endocrine cells, which include mainly three cell types: glucagon-secreting alpha-cells, insulin-producing beta-cells and SST-releasing delta-cells. SST is a powerful paracrine inhibitor of insulin and glucagon secretion (Svendsen and Holst, 2021). The regulation of SST secretion involves both intrinsic and paracrine mechanisms. There are pieces of evidence showing that SST expression in type 2 diabetes animal models is higher than the non-diabetic controls, and that there is a higher level of SST-like immunoreactivity after meal in T2D patients than healthy ones (Rorsman and Huising, 2018). However, there has been no surface marker reported for pancreatic delta-cells, impeding the purification of them from other pancreatic cell types. Here, we generated a reporter hESC line that expressed fluorescent mCherry to identify SST-producing delta-cells.

We applied the CRISPR/Cas9 knock-in system to insert the mCherry (Guo et al., 2023) to the C-terminus of SST in wild-type H1 cells, through transfection with lipofection with plasmids containing Cas9 encoding sequence and single guide RNA (sgRNA), which would form a U6-sgRNA-Cas9 complex and target the *SST*, and the other donor plasmid containing mCherry and puromycin resistance sequences, which was inserted between the homologous left arm (HL) and homologous right arm (HR) locating at the *SST* stop codon (Fig. 1A). Plasmids were transfected into H1 cells using a Thermo stem cell transfection reagent, including 1600 ng Cas9 plasmids and 900 ng donor plasmids (Fig. 1B). One day after transfection, 1 μg/ml puromycin was used for 5–7 days to select the puromycin resistant cells. Positive cells were then dissociated into single cells and seeded one cell per well in 96-well plates with puromycin selection. 5–7 days later, the colonies were detached and hand-picked using a microscope and then reseeded into 24-well plates for further expansion and genotyping. The PCR analysis using HL, HR and KI primers indicated successful CRISPR Knock-in, with resultant heterozygous mCherry insertion (Fig. 1C). The genome-edited cells showed a normal stem cell morphology (Fig. 1D). Sanger sequencing result confirmed the correct insertion of mCherry (Fig. 1E). Karyotyping analysis also verified the normal karyotype after genome-editing (Fig. 1F). The immunofluorescent staining results suggested that all the cells express the key stem cell marker proteins OCT4 and SOX2 (Fig. 1G). Additionally, quantitative PCR (qPCR) analysis proved that the expression of pluripotency marker genes *OCT4*, *NANOG* and *KLF4* in SST-mCherry cells was comparable to H1 wild-type cells (Fig. 1H), indicating that the differentiation potential of these cells was maintained. Immunostaining indicated that the reporter line maintained its ability to differentiate into all three germ layers (Fig. 1I).

SST-P2A-mCherry cells were differentiated into pancreatic endocrine

Table 1
Characterization and validation.

Classification (optional *italicized*)	**Output type**	**Result**	**Data**
Schematic of a transgene/ genetic modification	Schematic illustrating the structure and location of the introduced genetic modification	Representative edited allele and transgene structure	Fig. 1 panel A
Morphology	Photography	Typical hESC morphology	Fig. 1 panel D
Pluripotency status evidence for the described cell line	Qualitative analysis *(Immunocytochemistry)*	Positive for pluripotency markers: OCT4, SOX2	Fig. 1 panel G
	Quantitative analysis *(RT-qPCR)*	Positive for pluripotency markers: *OCT4*, *NANOG*, *KLF4*	Fig. 1 panel H
Karyotype	Karyotype (G-banding) and higher-resolution, array-based assays (KaryoStat, SNP, etc.)	46 XY, Resolution 550	Fig. 1 panel F
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	PCR specific to desired KI (junction sequencing)	Fig. 1 panel C
	Evaluation of the -(homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	Heterozygous genomic alteration	Fig. 1 panel C
	Transgene-specific PCR (when applicable)	ND	N/A
Verification of the absence of random plasmid integration events	PCR	PCR detection for plasmid backbones	N/A
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	All 21 STR loci tested matched	Submitted in the archive with journal
		ND	N/A
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR)	PCR specific to desired KI (junction sequencing)	Fig. 1 panel E
	PCR-based analyses	Heterozygous target integration	Fig. 1 panel C
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	ND	N/A
Off-target nuclease activity analysis	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	No off-target sites shown by PCR of genomic DNA and Sanger sequencing	Fig. S1
Specific pathogen-free status	Mycoplasma (RT-PCR)	Negative	Available with authors
Multilineage differentiation potential	STEMdiff™ Trilineage Differentiation Kit and directed differentiation of pancreatic delta-cell	SOX17 (endoderm), BRACHYURY (mesoderm), TUJ1 (ectoderm), SST (pancreatic delta-cell)	Fig. 1 panel I Fig. 1 panel J Fig. 1 panel K
List of recommended germ layer markers	Expression of these markers has to be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers	N/A	N/A

(*continued on next page*)

Table 1 (*continued*)

Classification (optional *italicized*)	Output type	Result	Data
	need to be shown per germ layer		
Outcomes of gene editing experiment (OPTIONAL)	Brief description of the outcomes in terms of clones generated/ establishment approach/screening outcomes	N/A	N/A
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	ND	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	ND	N/A
	HLA tissue typing	ND	N/A

organoids using a seven-step method (Rezania et al., 2014), which leads to development of functional mature pancreatic alpha-cells, beta-cells and a fraction of delta-cells from hESCs. At stage 7 day 14, planar cultured cells were dispersed and sorted by fluorescence-activated cell sorting (FACS). The proportion of mCherry-positive cells was accounted for 8.5 % (Fig. 1J). The sorted cells were then reseeded and immunofluorescent stained using a mouse anti-SST antibody to show the co-localization of mCherry with SST (Fig. 1K), indicating that mCherry positive cells are SST-expressing cells.

3. Materials and methods

3.1. Cell culture and differentiation

H1 hESCs and SST-P2A-mcherry cell line were maintained on Matrigel-coated (Thermo Scientific) plates with mTeSR1 (Stemcell Technologies) at 37 ℃ in 5 % CO_2, and medium was changed daily. The cells were with dissociated using 0.5 mM EDTA in DPBS and passaged every 5–7 days at a ratio of 1:50. Directed differentiation was performed using a seven-step protocol by T. Kieffer (Rezania et al., 2014).

3.2. Genome editing

The sgRNAs were designed by the CRISPOR webserver (https://crispor.tefor.net/). The sgRNA oligos were annealed and cloned into BbsI digested Px335 (addgene #42335) plasmids, according to the protocol from Dr. Feng Zhang' Lab. The sgRNA sequences are listed in Table 2. The donor plasmid (pUC57-SST-P2A-mcherry-NLS-puro) was modified based on pUC57 backbone. The HL and HR of SST were amplified from H1 cells by PCR and finally cloned into pUC57 vectors. A total of 800 ng sgRNA1-Cas9 plasmids, 800 ng sgRNA2-Cas9 plasmids, as well as 900 ng donor plasmids were transfected into 1×10^6 H1 cells using Lipofectamine Stem reagent (Thermo Scientific). After 24 h, puromycin (1 μg/ml) was used for 5–7 days to screen the resistant cells. Afterwards, the selected colonies were dissociated into single cells and reseeded into 96-well plates for further expansion and verification. The DNA was extracted using QuickExtract™ DNA Extraction Solution (Dakewe Biotech) to verify the correct targeting and insertion. PCR was performed using PrimeSTAR®GXL DNA Polymerase (TAKARA) by T100 Thermal Cycler (Bio-Rad). The PCR products were sequenced by Sangon Biotech (Shanghai, China). The primers used for sequencing analysis are shown in Table 2.

3.3. In vitro differentiation into three germ layers

The SST-P2A-mcherry cells were differentiated into the three germ layers: endoderm (SOX17), mesoderm (BRACHYURY), and ectoderm (TUJ1) *in vitro*, using STEMdiff™ Trilineage Differentiation Kit (STEMCELL) according to the manufacturer's instructions. The differentiated cells of three germ layers were stained and observed using a Carl Zeiss LSM980 confocal microscope (Zeiss).

3.4. Mycoplasma screening

PCR detection of Mycoplasma was conducted using a Taqman Mycoplasma Detection Kit (Geneseed).

3.5. RNA extraction and qRT-PCR

Total RNA was extracted by RNeasy Mini Kit (Qiagen) and reverse-transcribed into cDNA using Maxima H Minus reverse transcription reagent (Thermo Scientific), following which the quantitative PCR was performed using SYBR Premix Ex TaqII (TAKARA). The primers used are shown in Table 2.

3.6. Immunofluorescence staining

Cells were seeded onto Matrigel-coated circular glass slides in 24-well plates. After 4 h, the glass slides were washed three times with PBS and fixed with 4 % paraformaldedyde for 30 min. Cells were permeabilized with PBS containing 0.5 % Triton X-100 at room temperature (RT) for 20 min, and then blocked with 5 % donkey serum for 30 min. After three times washing with PBS, the cells were stained with primary antibodies overnight at 4 ℃, washed three times with PBS and then incubated with the secondary antibodies for 2 h at RT. DAPI (Thermo Scientific) was stained together with secondary antibodies. The stained samples were washed by PBS and imaged using a Carl Zeiss LSM980 confocal microscope (Zeiss).

3.7. FACS

Differentiated cells at stage 7 day 14 were dissociated into single cells using Accutase (Stemcell), and then resuspended in sorting buffer containing 0.5 % BSA and 2 mM EDTA. FACS was performed by Sony MA900 flow cytometry instrument (Sony).

3.8. Off-target analysis

The CRISPR RGEN Tool (http://www.rgenome.net/cas-offinder/) was used to predict the most likely off-target sites. The predicted regions of genomic DNA were amplified using PrimeSTAR®GXL DNA Polymerase (TAKARA) and sequenced (Sangon Biotech).

3.9. Karyotyping and short tandem repeat (STR) analysis

Chromosome analysis was checked by Biowing Applied Biotechnology (Shanghai, China). The STR analysis was performed by IGE Biotechnology (Guangzhou, China).

CRediT authorship contribution statement

Tongran Zhang: Writing – original draft, Software, Methodology, Investigation. **Feng Zhang:** Methodology, Investigation. **Nannan Wang:** Validation, Software. **Tao Xu:** Resources, Conceptualization. **Lingqiang Zhu:** Writing – review & editing, Supervision. **Lihua Chen:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Huisheng Liu:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

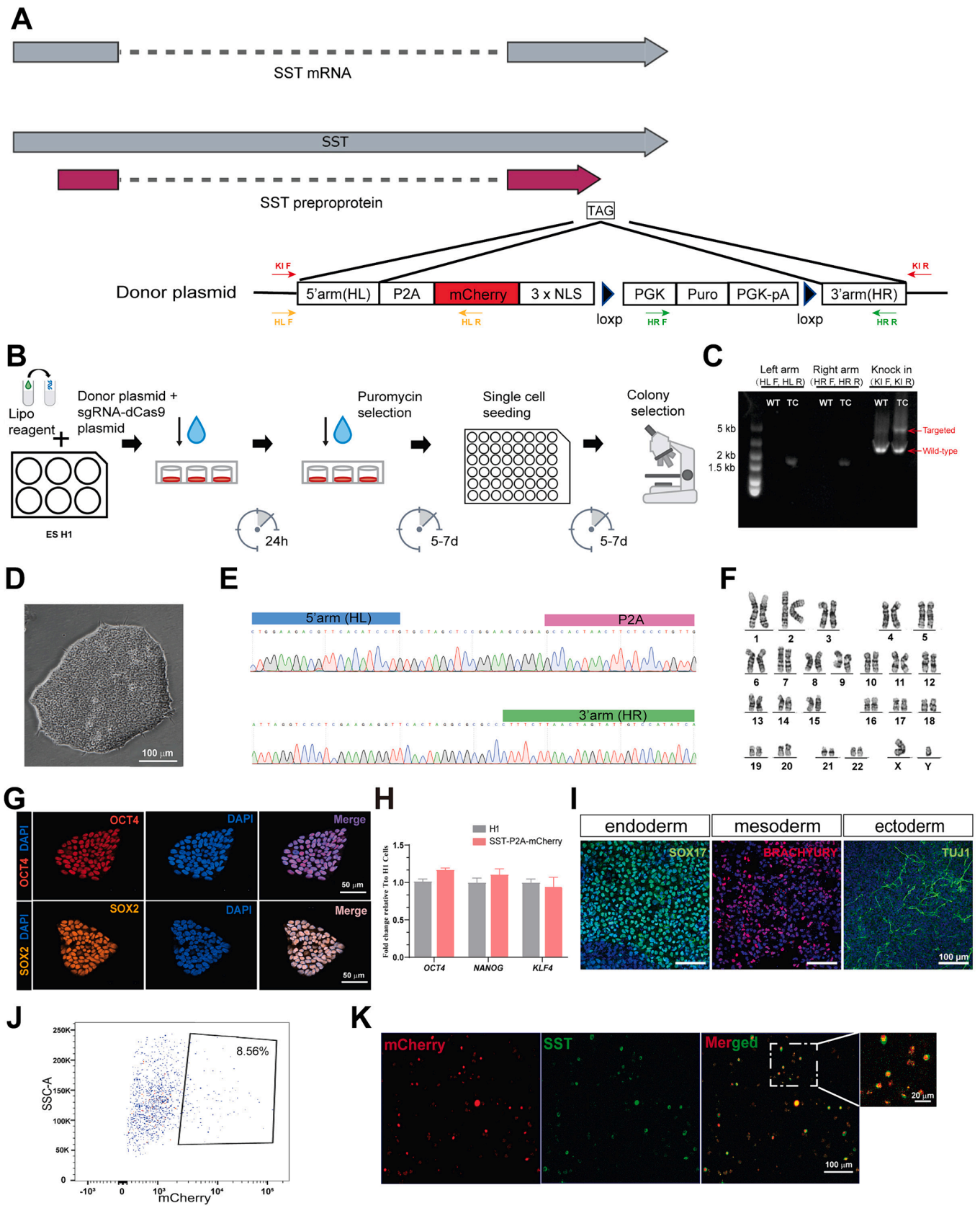
A
SST mRNA
SST
SST preproprotein
TAG
Donor plasmid
KI F
5'arm(HL)
P2A
mCherry
3 x NLS
loxp
PGK
Puro
PGK-pA
loxp
3'arm(HR)
KI R
HL F
HL R
HR F
HR R
B
Lipo reagent
Donor plasmid + sgRNA-dCas9 plasmid
ES H1
24h
Puromycin selection
5-7d
Single cell seeding
Colony selection
5-7d
C
Left arm (HL F, HL R)
Right arm (HR F, HR R)
Knock in (KI F, KI R)
WT TC WT TC WT TC
5 kb
2 kb
1.5 kb
Targeted
Wild-type
D
100 μm
E
5'arm (HL)
P2A
3'arm (HR)
F
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y
G
OCT4
DAPI
Merge
SOX2
50 μm
H
H1
SST-P2A-mCherry
Fold change relative Tto H1 Cells
1.5
1.0
0.5
0.0
OCT4
NANOG
KLF4
I
endoderm
mesoderm
ectoderm
SOX17
BRACHYURY
TUJ1
100 μm
J
250K
200K
150K
100K
50K
0
SSC-A
8.56%
-10³ 0 10³ 10⁴ 10⁵
mCherry
K
mCherry
SST
Merged
20 μm
100 μm

Fig. 1. xxxx

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Rabbit anti-OCT4	1:200	Abcam Cat# ab19857, RRID: AB_445175
	Rabbit anti-SOX2	1:1000	Abcam Cat# ab97959, RRID: AB_2341193
Differentiation Markers	Mouse anti-SST	1:200	BD Biosciences Cat# 566031,
	Mouse anti-SOX17	1:200	RRID:AB_2739475
	Rabbit anti-BRACHYURY	1:1000	Abcam Cat#ab84990, RRID: AB_1861437
	Rabbit anti-TUJ1	1:500	Abcam Cat# ab209665, RRID:AB_2750925
			Abcam Cat#ab18207, RRID: AB_444319
Secondary antibodies	Alexa Fluor 546 anti-Rabbit IgG	1:500	Thermo Fisher Scientific Cat# A10040, RRID:
	(H + L)	1:500	AB_2534016
	Alexa Fluor 647 anti-Rabbit IgG	1:500	Thermo Fisher Scientific Cat# A-31573, RRID:
	(H + L)	1:500	AB_2536183
	Alexa Fluor 488 anti-Mouse IgG		Molecular Probes Cat# A-21202, RRID:
	(H + L)		AB_141607
	Alexa Fluor 488 anti-Rabbit IgG		Invitrogen Cat#A21206,
	(H + L)		RRID:AB 2,535,792
Nuclear stain	DAPI	1:1000	Thermo Fisher Scientific Cat#622492
Site-specific nuclease			
Nuclease information	SpCas9		
Delivery method	Lipofection		
Selection/enrichment strategy	Puromycin		

Primers and Oligonucleotides used in this study		
	Target	**Forward/Reverse primer (5′-3′)**
Episomal Plasmids (qPCR or RT-PCR)	N/A	N/A
Pluripotency Markers (qPCR)	*OCT4*	CCGAAAGAGAAAGCGAACCAG/
	NANOG	ATGTGGCTGATCTGCTGCAGT
	KLF4	AATGGTGTGACGCAGGGATG/
		GGACTGGATGTTCTGGGTCTG
		GATGATGCTCACCCCACCTT/GGCGAATTTCCATCCACAGC
House-Keeping Genes (qPCR)	GAPDH	TGCACCACCAACTGCTTAGC/
		GGCATGGACTGTGGTCATGAG
Genotyping (desired allele/transgene presence detection)	KI(5.2 kb) WT(2.0 kb)	CTAAGCCTTGCTCCTGCCCCATTGG/
	HL(1.6 kb)	GAGCTCCAATACCTGCAATATAG
	HR(1.6 kb)	CTAAGCCTTGCTCCTGCCCCATTGG/
		GTAGTGGCCGCCGTCCTTCAGCTTCAGC
		GAAGTAGCACGTCTCACTAGTCTCGTGC/
		CTAATGCAAGGGTCTCGCTGAAG
Targeted mutation analysis/sequencing	Junction	CTAAGCCTTGCTCCTGCCCCATTGG/
		GAGCTCCAATACCTGCAATATAG
Potential random integration-detecting PCRs	Plasmid backbone (Ampicillin	GATCAGTTGGGTGCACGAGTG/
	region)	TGCTACAGGCATCGTGGTGTC
gRNA oligonucleotide/crRNA sequence	sgRNA1	GGCTAACTCAAACCCGGCTA
	sgRNA2	GACTAGTTAAGAAAGCTAAC
Genomic target sequence(s)	*SST*	NCBI reference sequence (NC_000003.12) Exon 2
Bioinformatic gRNA on- and -off-target binding prediction tool used, specific sequence/outputs link(s)	CRISPR RGEN Tools	https://www.rgenome.net/cas-offinder/result?hash=9760fcf15dc5e3141704d8e8fa4722f6
Primers for top off-target mutagenesis predicted site sequencing (for all CRISPR/Cas9, ZFN and TALENs)	OT1 (LINC01933) (408 bp)	CTTCTGCTACTGTGTCAAGCAG/
	OT2 (NC000004.12) (413 bp)	GAGCAGGCAAGGTTGTCTAGCCAG
	OT3 (NC000012.12) (406 bp)	GCATGCTGATGACTTCACTGTATG/
	OT4 (NC000001.11) (487 bp)	GATGGTCAATGAGAGTGGACTG
	OT5 (NC000010.11) (480 bp)	TAAGGAGGCAGCTTTAGGCT/
		CTCCATCCACTGTCTGCCTAGTTAC
		CATGACACAGCCTGCCCTGATAG/
		CAGAGTGGAACGGTTCTCTGTGG
		CCTGGTACACTTGCTGGGAACGTA/
		CCAAGCTCCTGATAACCATC
ODNs/plasmids/RNA molecules used as templates for HDR-mediated site-directed mutagenesis.	N/A	N/A

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103397.

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