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Generation of a fluorescent mNeonGreen insulin reporter line in the H1 (WA01) hESC background

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Abstract

Over the past decade, the use of human stem cell-derived β cells (SC-β cells) to model pancreatic β cell development, function and disease has become increasingly common. Though protocols are rapidly improving, current directed differentiation strategies do not yield a pure population of insulin-positive SC-β cells *in vitro*. Therefore, it is experimentally advantageous to have reporter lines that allow for live sorting of insulin-positive populations. To aid in these studies, we have knocked mNeonGreen fluorescent protein into the endogenous insulin locus of the commonly used H1 (WA01) human embryonic stem cell line.

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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.

CRediT authorship contribution statement

Karla F. Leavens: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Catherine Osorio-Quintero:** Formal analysis, Methodology. **Elisabeth Ashlyne Yeuteuh:** Data curation, Formal analysis, Investigation, Methodology. **Francesca T. Perez-Profeta:** Data curation, Formal analysis, Investigation, Methodology. **Anna Ada Dattoli:** Formal analysis, Methodology. **Fabian L. Cardenas-Diaz:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Deborah L. French:** Conceptualization, Supervision. **Paul Gadue:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103559.

1. Resource utility

This reporter line expresses the fluorescent protein mNeonGreen under the control of the endogenous insulin promoter and allows for live visualization and isolation of human stem cell-derived β cells expressing insulin, aiding in the study of β cell development, function and disease modeling.

2. Resource details

As protocols for the directed differentiation of human embryonic stem cells (hESC) into pancreatic β cells do not result in a homogenous population, reporter lines have been generated to allow for the isolation of insulin-positive stem cell-derived β cells (SC-β cells). One widely used line is the Mel1 $Ins^{GFP/w}$ (Mel1 INS-GFP) hESC reporter line, published in 2012 (Micallef et al., 2012), in which GFP expression is driven by one of the endogenous insulin loci (*INS*). The H1 (WA01) line is another commonly used hESC line that generates a high percentage of insulin-positive cells (Cardenas-Diaz et al., 2019). Using CRISPR/Cas9, we have inserted mNeonGreen, a monomeric fluorescent protein with similar excitation/emission spectra to GFP but with significantly increased brightness and stability (Shaner et al., 2013; Tanida-Miyake et al., 2018), into one allele of the endogenous *INS* locus. The targeting strategy employed and resulting reporter knock-in are illustrated in Fig. 1A. To aid with knock-in efficiency and protein processing, we designed mNeonGreen cDNA to follow preproinsulin cDNA through the middle of the C peptide sequence (PPImNG) as previously done using GFP and mouse proinsulin (Watkins et al., 2002). This could potentially allow for targeting of mNeonGreen protein to the insulin secretory vesicles but minimize the likelihood of a misfolded insulin-mNeonGreen fusion protein that could induce endoplasmic reticulum stress. The targeting vector contained the partial preproinulin cDNA-mNeonGreen construct and an antibiotic neomycin cassette driven by the PGK promoter and flanked by loxP sites. Left and right arms of homology (LAOH and RAOH) to the endogenous *INS* locus facilitated integration of the construct by CRISPR-Cas9 gene editing. PCR screening was used to detect the integration of the targeting vector into the *INS* locus on both sides of the construct and targeted clones were screened for the presence of the WT allele to verify they were heterozygous (Fig. 1B). Sequencing was performed on all PCR products to ensure no indels were present. The final isolated clone line was then characterized as below (Table 1, Table 2).

The H1 INS-PPImNG line exhibited normal hESC morphology (Fig. 1C). Expression of pluripotency markers by immunofluorescence and gene expression was normal (Supplementary Figure 1A and C). Flow cytometry showed normal expression of stem cell markers (Supplementary Figure 1B). Karyotype of the line was normal (Supplementary Figure 1D) and DNA fingerprinting by STR analysis confirmed the identity of PPImNG-targeted line to the unmodified H1 (WA01) line (data available with authors). The line was successfully differentiated into all 3 germ cells layers (Fig. 1D, E and Supplementary Figure 1E). PCR screening confirmed there was no integration of the reprograming plasmids (Supplementary Figure 1F). The archived cell stock tested negative for Mycoplasma contamination by PCR (Supplementary Figure 1G). The H1 INS-PPImNG line was differentiated in SC-β cells using aggregate culture protocols as previously described

(Cardenas-Diaz et al., 2019), and the cells differentiate with similar yields and results as the parental H1 line. mNeonGreen is first visible by light and confocal microscopy around day 17, reaching maximal brightness around day 30 (Fig. 1F and G). Flow cytometry showed good correlation of cells expressing C peptide and mNeonGreen (Fig. 1E), demonstrating that this reporter line will allow for live cell sorting of insulin-positive SC-β cells.

3. Materials and methods

3.1. Generation of targeted H1 INS-PPImNG hESC line

Cells were plated on DR4-MEFs 24 h pre-transfection. Quantities used for transfection: Cas9-GFP 0.1 μg, gRNA 0.1 μg, targeting vector 1.8 μg, and 3 μl Lipofectamine™ Stem Transfection Reagent (Maguire et al., 2019). Neomycin selection (40 μg/ml) was done day 2–12 post-transfection. Colonies were then picked and expanded for screening. DNA was isolated using Purelink Genomic DNA extraction kit (ThermoFisher) and targeted clones were identified using PCR screening. PCR products were sequenced by Genewiz (Azenta Life Sciences) and analysed using SnapGene software.

3.2. hESC culture, pluripotency evaluation and differentiation

hESCs were maintained on irradiated MEFs, grown in 37 °C with 5 % CO_2 and 5 % O_2 using media of DMEM/F12 (Corning) supplemented with 20 % knock-out serum replacement (ThermoFisher), 100 μM nonessential amino acids, 2 mM glutamine, mercaptoethanol, and 10 ng/mL bFGF (R&D Systems). Cells were split every 3–4 days once reaching 80–90 % confluence at a ratio of 1:12 using TrypLE dissociation reagent (Gibco) and replated with 10 μM Rock-inhibitor Y27632 dihydrochloride (Tocris). Pluripotency evaluation and germ layer differentiations were performed on passage 12–15 cells after gene editing. Differentiation of hESCs into pancreatic β cells was performed as described previously (Cardenas-Diaz et al., 2019). Mesoderm and ectoderm differentiations were performed using standard in-lab protocols (details available with authors); expression of HAND1 and CD31 at day 4 for mesoderm and SOX1 and FOXG1 at day 7 for ectoderm were analysed by FACS.

3.3. Extracellular and intracellular staining by flow cytometry

Trypsin-dissociated cells were analysed using a CytoFLEX flow cytometer (Beckman Coulter) and FlowJo software program. Expression of extracellular proteins was evaluated in live cells using antibodies diluted in FACS buffer (Wilken et al., 2023). Expression of intracellular proteins was evaluated following fixation in 1.6 % paraformaldehyde using antibodies diluted in saponin for permeabilization (BioLegend PermWash). Cells were incubated with antibodies at 37 °C for 30 min.

3.4. RealTime qPCR

RNA was isolated using the PureLink RNA Mini Kit (ThermoFisher) and qRT-PCR was performed as previously described (Maguire, 2019). Expression of pluripotency genes in H1 INS-PPImNG cells at passage 12–15 post-gene editing was compared to parental H1 hESC line and to H1 SC-β cells.

3.5. Imaging

Immunofluorescence for pluripotency markers was performed in hESCs as previously described (Maguire, 2019) and imaged using an EVOS cell imaging system (ThermoFisher). Aggregates from pancreas differentiation at day 23 were fixed in 4 % paraformaldehyde, washed in PBS+0.2 % Triton X-100, stained with DAPI, washed, and mounted in SlowFade Gold antifade reagent (Invitrogen). Images were taken using a Leica TCS SP8 with 40 × NA 1.3 oil objective, 115 μm confocal pinhole, and HyVolution super-resolution. Samples were sequentially excited for DAPI and mNeonGreen using standard filters (Chroma) with 1 μM Z-step size.

3.6. Karyotyping, STR analysis, and CNV analysis

Chromosomal G-band analysis and STR analysis were performed by Cell Line Genetics, Inc. (Madison, WI) at passage 14 and 16 post-gene editing, respectively. For karyotyping, 20 metaphases were counted, and 7 were analysed with fair resolution. Copy number variation analysis was performed at the Children's Hospital of Philadelphia Center for Applied Genomics.

3.7. Mycoplasma testing

Mycoplasma testing was performed by PCR as previously described (Wilken et al., 2023; Maguire, 2019).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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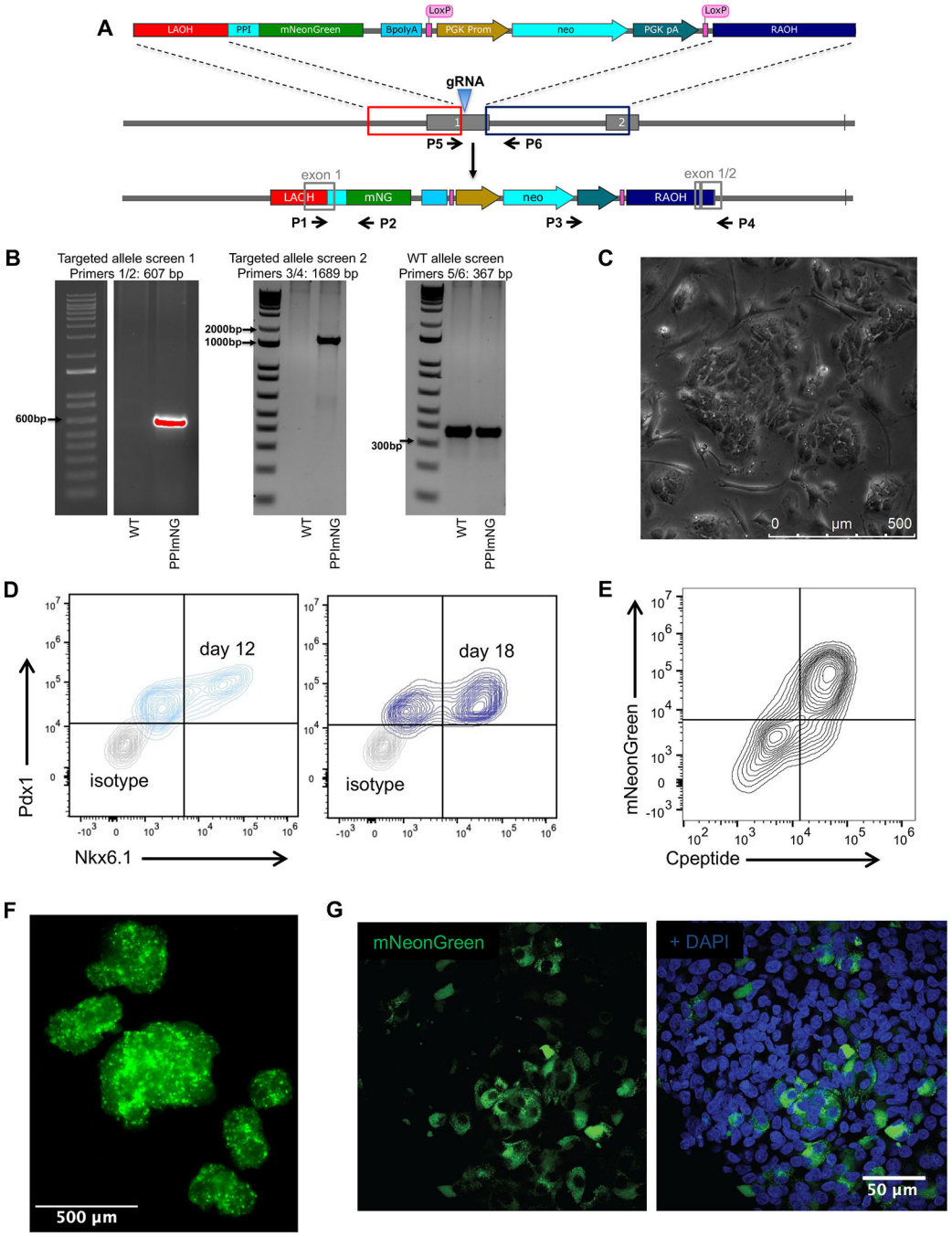

Fig. 1.

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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	A visual representation of the targeting strategy and resulting mNeonGreen reporter knocked into the endogenous insulin allele	Fig. 1A
Morphology	Photography	Normal	Fig. 1C
Pluripotency status evidence for the described cell line	Immunofluorescence	Positive staining of pluripotency markers NANOG, OCT3/4, SOX2	Supplemental Figure 1A
	Flow cytometry	SSEA3/4 = 97.2 %	Supplemental Figure 1B
	RT-qPCR	TRA-1–60/81 = 97.5 % DNMT3B, NANOG, OCT4, SOX2	Supplemental Figure 1C
Karyotype	Karyotype (G-banding)SNP Microarray	46XY, Fair ResolutionNo significant genomic alterations compared with parental line detected	Supplemental Figure 1D Data available with authors
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	PCR confirming targeted allele with transgenic construct, 2 sites	Fig. 1B
	Evaluation of the heterozygous status of introduced genomic alteration(s)	PCR confirming heterozygous wildtype allele	Fig. 1B
	Transgene-specific PCR (when applicable)	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	No integration of reprogramming plasmids	Supplemental Figure 1F
Parental and modified cell line genetic identity evidence	STR analysis	Matched ESCs to parental line	Supplementary file, submitted in the archive with journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Heterozygous transgene present in endogenous insulin locus	Sanger sequencing available with authors
	PCR-based analyses	PCR confirming targeted allele with transgenic construct and heterozygous wildtype allele	Fig. 1B
	Flow CytometryImmunofluorescence	Co-staining of C-peptide and mNeonGreen by flow cytometryVisualization of mNeonGreen by fluorescent and confocal microscopy	Fig. 1EFig. 1F and G
Off-target nuclease activity analysis	PCR/sequencing analysis across top 5 predicted off-target sites	No mutations detected	Sanger sequencing available with authors
Specific pathogen-free status	Mycoplasma	Mycoplasma negative by PCR	Supplemental Fig. 1G
Multilineage differentiation potential	Directed differentiation to endoderm *in vitro*	Co-staining of PDX1 and Nkx6.1 by flow	Fig. 1D
	Directed differentiation to pancreatic β-cell *in vitro*	cytometry	Fig. 1E
	Directed differentiation to ectoderm *in vitro*	Co-staining of C-peptide and mNeonGreen by	Supplemental Figure 1E

Classification (optional *italicized*)	Output type	Result	Data
	Directed differentiation to mesoderm *in vitro*	flow cytometry Quantification of Sox1 and FOXG1 protein by flow cytometry Quantification of Hand1 and CD31 protein by flow cytometry	Supplemental Figure 1E
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 need to be shown per germ layer	As above	
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	N/A	N/A
Genotype – additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

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Table 2

Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers (Flow cytometry)	SSEA-3	1:50	Biolegend, Cat# 330306, RRID: AB_1279440
	SSEA-4	1:400	Biolegend, Cat# 330408, RRID: AB_1089200
	Tra-1–60	1:50	Biolegend, Cat# 330614, RRID: AB_2119064
	Tra-1–81	1:50	Biolegend, Cat# 330706, RRID: AB_1089242
Pancreatic endocrine (Flow cytometry)	PDX1-biotinylated	1:50	R&D, Cat# BAF2419, RRID:AB_416757
	NKX6.1	1:300	DSHB#F55A10-c, RRID:AB_2631146
Mesoderm (Flow cytometry)	C-peptide	1:100	Cell Signaling, Cat# sc-4593S, RRID: AB_10691857
	Hand1	1:200	Novus, Cat# NBP2-00576, RRID: AB_2877685
Ectoderm (Flow cytometry)	CD31-FITC conjugated	1:100	BioLegend Cat# 303118, RRID: AB_2247932
	Sox1-PE conjugatedFOXG1	1:201:300	BD, Cat# 561592, RRID:AB_10714631Abcam, Catalog #ab196868, RRID: AB_2892604
Sternness Markers (ICC)	NANOG	1:400	Cell Signaling, Cat# 4903S, RRID: AB_10559205
	OCT3/4	1:200	SCBT, Cat# sc-5279, RRID:AB_628051
	SOX2	1:400	Cell Signaling, Cat# 3579S, RRID: AB_2195767
Secondary antibodies	Goat anti-mouse IgG-AF488	1:400	Thermo Fisher Scientific, Cat# A-11029, RRID: AB_2534088
	Goat anti-rabbit IgG-AF488	1:400	Thermo Fisher Scientific, Cat# A-11008, RRID: AB_143165

	Goat anti-mouse IgG-647	1:1000	Jackson, Cat# 115-605-207, RRID:AB 2338918
	Goat anti-rabbit-647SA-488	1:1000 1:1000	Jackson, Cat# 111-605-144, RRID:AB_2338078 Jackson, Cat# 016-540-084
Nuclear stain	DAPI		Vectashield Antifade mounting media with DAPI, Vector Laboratories catalog # H-1200-10

Site-specific nuclease

Nuclease information	Cas9-GFP	pCas9-GFP (Addgene #44719): pCas9_GFP was a gift from Kiran Musunuru (Addgene plasmid # 44719; https://n2t.net/addgene:44719; RRID:Addgene_44719)
Delivery method	Lipofection	Plasmid transfection using Lipofectamine™ Stem Transfection Reagent (3 μL/well)
Selection/ enrichment strategy	Neomycin selection	40 μ/mL × 10 days

Primers and Oligonucleotides used in this study

	Target	**Forward/Reverse primer (5'–3')**
Pluripotency Markers (qPCR)	*DNMT3B* *NANOG* *OCT4* *SOX2*	TACAGACGTGTGCAGTTGTAGGCA/ GTGCAGACTCCAGCCCTTGTATTT CCTGAAGACGTGTGAAGATGAG/ GCTGATTAGGCTCCAACCATAC AACCTGGAGTTTGTGCCAGGGTTT/ TGAACTTCACCTTCCCTCCAACCA ATGACCAGCTCGCAGACCTACA/ GGACTTGACCACCGAACCCA
House-Keeping Gene (qPCR)	*TBP*	TTGCTGAGAAGAGTGTGCTGGAGATG/ CGTAAGGTGGCAGGCTGTTGTT
Targeted mutation analysis/ sequencing	Targeted allele screen 1 (primers 1, 2) Targeted allele screen 2 (primers 3, 4) Wildtype allele screen (primer 5, 6)	GGCCATCAAGCAGGTCTGTTC/ GAGAACTGGAGGTCACCCTT GCAGCGCATCGCCTTCTATC/ AGGGCTGGTTCAAGGGCTTTATTC TCTCCCAGATCACTGTCCTT/TTAGGACGTGACCAAGAGAAC
Mycoplasma detection (PCR)	16S ribosomal RNA (518 bp) GADPH internal control (153 bp)	CGCCTGAGTAGTACGTTCGC/GCGGTGTGTACAAGACCCGA GTGGACCTGACCTGCCGTCT/GGAGGAGTGGGTGTCGCTGT
gRNA oligonucleotide		CCTGTGGATGCGCCTCCTGCCCC

Genomic target sequence	Genomic sequence chr11:2160943–2160966:	TTCCCCGCACACTAGGTAGAGAGCTTCCACCAGGTGTGAGCCGCACAGGTGTTGGTTCACAAAGGCTGCGGCTGGGTCAG GTCCCCAGAGGGCCAGCAGCGCCAGCA**GGGGCAGGAGGCGCATCCAC**<u>**AGG**</u>GCCATGGCAGAAGGACAGTGATCTGGGAGA CAGGCAGGGCTGAGGCAGGCTGAAGGCCAGGTGCCCTGCCTTGGGGCCCCTGGGCTCACCCCCACATGCTTCACGAGCCC AGCCACGTCCTCCCTGCTGCAGAGCTGGGGCCTGGGGTCCAGCCACCCTGGAATCCTGAGCCCACCTGACGCAAAGGCCC TTGGAACAGACCTGCTTGATGGCCTCTT
Potential random integration-detecting PCRs	Cas9 Plasmid Targeting vector gRNA vector	ATAATACCGCGCCACATAGC/ CGGCATCAGAGCAGATTGTA ATAATACCGCGCCACATAGC/ GTGGACTCTTGTTCCAAACTGG TGTAAAACGACGGCCAGT/ CAGGAAACAGCTATGAC
Bioinformatic gRNA on– and –off-target binding prediction tool used	CRISPOR	https://crispor.tefor.net/crispor.py?batchId = 5D8WTOgpLmOto0GK2zU5
Primers for top off-target mutagenesis predicted site sequencing	OT1: intron_CFI_chr4_109768664	CAATGCTGTCTCCCTGTCCC/ TTTTGTCTGCCCCAGCTTGA
	OT2: intergenic_SLC26A9| RAB7B_chr1_205956753	CCAGACCTAGGCTCCCTTCT/ ACCTAACTGTGGTTAAGATGAAGA
	OT3: intergenic_RP11-351C8.1| PCAT1_chr8_126902640	AGTCACCTTTTAGAAGCCAGAAGT/ CCTCTCACGGTGGTATTGGG CGGGTGTTGGTAGCAAAAGG/
	OT4: exon_TTN_chr2_178701172	ACTGAAAGAAATGCATGAGACTTT AGACTCAGTTTGTATGCTGGCA/TCCCTGCATCACTTCTTTCCT
	OT5:intergenic_RRAGB| RP13-188A5.1_chrX_55803007	

Resource Table

Unique stem cell line identifier	WAe001-A-2I
Alternative name(s) of stem cell line	CHOPe004-A-2I H1 INS-PPImNG
Institution	The Children's Hospital of Philadelphia, Philadelphia, PA USA
Contact information of the reported cell line distributor	Karla F. Leavens, leavensk@chop.edu
Type of cell line	ESC
Origin	Human
Additional origin info (applicable for human ESC or iPSC)	Age: N/A Sex: M Ethnicity if known: N/A
Cell Source	WAO1 (H1)
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	MEFs
Type of the Genetic Modification	Reporter knock-in
Associated disease	N/A
Gene/locus modified in the reported transgenic line	Insulin (INS), 11p15.5
Method of modification / user-customisable nucleases (UCN) used, the resource used for design optimisation	CRISPR/Cas9 https://crispor.gi.ucsc.edu/
User-customisable nuclease (UCN) delivery method	Plasmid transfection using Lipofectamine™ Stem Transfection Reagent
All double-stranded DNA genetic material molecules introduced into the cells	Cas9-GFP plasmid, targeting vector plasmid, gRNA plasmid
Evidence of the absence of random integration of any plasmids or DS DNA introduced into the cells.	PCR from genomic DNA for plasmid backbone
Analysis of the nuclease-targeted allele status	PCR/sequencing of the targeted allele and the untargeted wildtype allele
Homozygous allele status validation	N/A, heterozygous reporter knock-in line
Method of the off-target nuclease activity prediction and surveillance	Targeted PCR/sequencing
Descriptive name of the transgene	Preproinsulin (partial cDNA)-mNeonGreen fusion
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	Neomycin
Inducible/constitutive expression system details	N/A
Date archived/stock creation date	2023
Cell line repository/bank	https://hpscreg.eu/cell-line/WAe001-A-2I
Ethical/GMO work approvals	The parental cell line H1 (WA01) is on the NIH Human Embryonic Stem Cell Registry with NIH approval number NIHhESC-10–0043. All research involving hPSC is approved by The Children's Hospital of Philadelphia IRB (approval 09–007042)
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pCas9-GFP (Addgene #44719): pCas9_GFP was a gift from Kiran Musunuru (Addgene plasmid # 44719; https://n2t.net/addgene:44719; RRID: Addgene_44719) gRNA Cloning Vector (Addgene #41824): gRNA_Cloning Vector was a gift from George Church (Addgene plasmid # 41824; https://n2t.net/addgene:41824; RRID:Addgene_41824)