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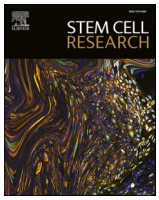

Generation of ARX-T2A-H2B-CFP x C-PEP-mCherry-hiPSC double reporter line for monitoring of pancreatic differentiation

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A B S T R A C T

Pancreatic islets consist of several different endocrine cell types that work in harmony. Aside from primary pancreatic islets, stem cell-derived pancreatic islets can be used as an alternative research and disease model. Here, we introduce a double reporter line of ARX-T2A-H2B-CFP x C-PEP-mCherry-hiPSC through CRISPR/Cas9-mediated insertion of mCherry in the C-terminus of C-Peptide in the previously published ARX-CFP hiPSC line. This reporter line allows live monitoring of stem cell-derived pancreatic alpha and beta cells throughout differentiation.

1. Resource table

Unique stem cell line identifier	HMGUi001-A-54
Alternative name(s) of stem cell line	ARX-T2A-H2B-CFP x C-PEP-mCherry-hiPSC; ARX-CFP x C-PEP-mCherry; $ARX^{CFP/CFP}$ x $C\text{-}PEP^{mCherry/+}$
Institution	Institute of Diabetes and Regeneration Research, Helmholtz Munich, 85764 Neuherberg, Germany
Contact information of the reported cell line distributor	Heiko Lickert, heiko.lickert@helmholtz-munich.de
Type of cell line	iPSC
Origin	Human, HMGUi001-A-4 hiPSC described in Moya et al., 2020
Additional origin info*(applicable for human ESC or iPSC)*	Age: N/A Sex: Female Ethnicity: Caucasian
Cell Source	Fibroblast
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	Feeder free
Type of the Genetic Modification	Transgene generation – Heterozygous insertion of fluorescent reporter
Associated disease	N/A
Gene/locus modified in the reported transgenic line	Insulin gene (INS)/11p15.5

(*continued on next column*)

(*continued*)

Unique stem cell line identifier	HMGUi001-A-54
Method of modification / user-customisable nucleases (UCN) used, the resource used for design optimisation	CRISPR/Cas9
User-customisable nuclease (UCN) delivery method	Lipofectamine transfection
All double-stranded DNA genetic material molecules introduced into the cells	pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA; Addgene #86986 pBlueScript Ins-C-pep-mCherry Fusion; (Siehler et al., 2021)
Evidence of the absence of random integration of any plasmids or DS DNA introduced into the cells.	PCR of plasmid backbone
Analysis of the nuclease-targeted allele status	PCR and Sequencing of the heterozygous targeted allele; PCR and Sequencing of untargeted allele.
Homozygous allele status validation	N/A
Method of the off-target nuclease activity prediction and surveillance	Targeted off-target site sequencing
Descriptive name of the transgene	ARX-T2A-H2B-CFP-Flag x C-Peptide-mCherry
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	No selection cassette used
Inducible/constitutive expression system details	N/A

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(*continued*)

Unique stem cell line identifier	HMGUi001-A-54
Date archived/stock creation date	January 17, 2023
Cell line repository/bank	https://hpscreg.eu/cell-line/HMGUi001-A-54
Ethical/GMO work approvals	The choice of appropriate human donors, the procedures for skin biopsy, isolation of dermal fibroblasts, generation of iPSCs and their use in further scientific investigations were performed under the positive votes of the Ethics Committee of the Medical Faculty of the Eberhard Karls University, Tübingen (file numbers 629/2012BO2 and 130/2018BO2) and of the Medical Faculty of the Technical University Munich (file number 219/20 S). The study design followed the principles of the Declaration of Helsinki. All study participants gave informed consent prior to entry into the study.
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA was a gift from Ralf Kuehn (Addgene plasmid # 86,986; https://n2t.net/addgene:86986; RRID:Addgene_86986)

2. Resource utility

The ARX-T2A-H2B-CFP x C-PEP-mCherry-hiPSC (HMGUi001-A-54) line is a double reporter line marking pancreatic alpha cells, alpha progenitors (ARX^{+}) and pancreatic beta cells ($C\text{-}PEP^{+}$). The reporter expression allows live monitoring of differentiation efficiency and sorting specific cell populations, aiding in the in-depth characterisation of pancreatic alpha and beta cells. See (Table 1).

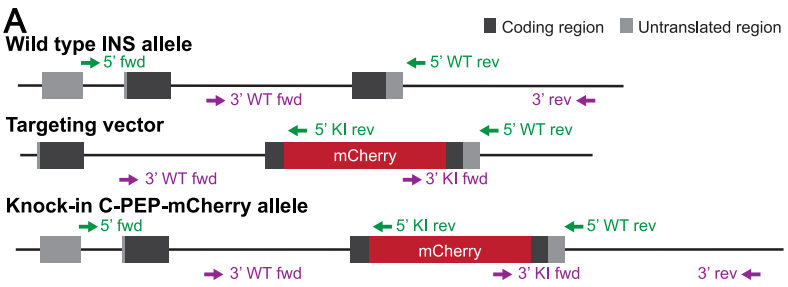
3. Resource details

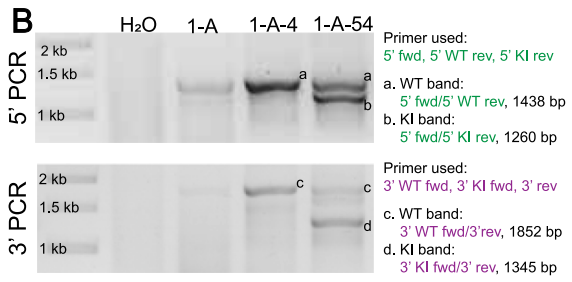
Diabetes mellitus is one of the most common and fastest-growing metabolic diseases, with an estimated 537 million people worldwide suffering from it in 2021 (Sun et al., 2022). People with diabetes have difficulties regulating their blood glucose levels due to impairment in insulin sensitivity or the loss of insulin-producing cells located in the pancreatic islets. Pancreatic islets are composed of several endocrine cell types which secrete hormones to regulate blood glucose levels. The two main hormones regulating blood glucose are insulin and glucagon, secreted from pancreatic beta cells and alpha cells, respectively. Studies on these endocrine cells are crucial for diabetes research. Aside from primary islets, stem cell-derived islets (SC-islets) can be used as an alternative research and disease model. To study the interaction between alpha and beta cells, we have generated the double reporter hiPSC line.

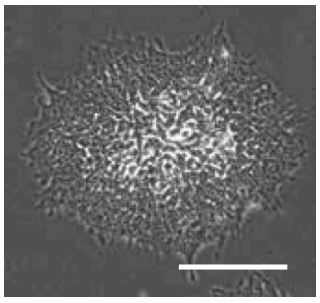
The ARX-T2A-H2B-CFP x C-PEP-mCherry-hiPSC (HMGUi001-A-54) was generated with a previously published strategy (Siehler et al., 2021). In short, the HMGUi001-A-4 ($ARX^{nCFP/nCFP}$ (Moya et al., 2020)) line was targeted with heterozygous insertion of mCherry at the C-terminus of C-Peptide located in the Exon 3 of the insulin locus (Fig. 1A). Correct insertion was confirmed through 5′ and 3′ PCR (Fig. 1B) and sequencing of both alleles showed correct insertion in one allele and no unwanted indels (insertion or deletions) in the wildtype allele around

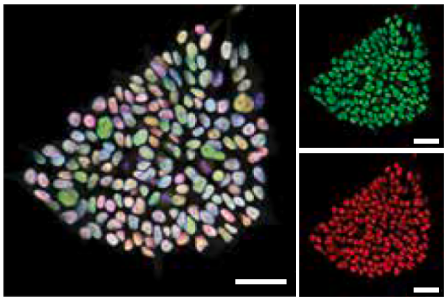
Table 1
Characterization and validation.

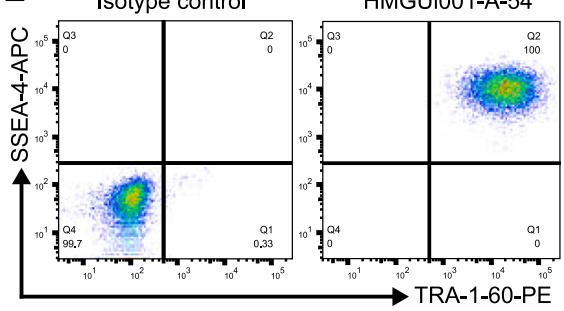
Classification	Output type	Result	Data
Schematic of a transgene/genetic modification	Schematic illustrating the structure and location of the introduced genetic modification		Fig. 1A
Morphology	Brightfield imaging	Normal morphology	Fig. 1C
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Staining for OCT3/4 and SOX2	Fig. 1D
	Quantitative analysis (Flow cytometry)	TRA-1–60 and SSEA-4 (>90 %)	Fig. 1E
Karyotype	Karyotype (G-banding)	46, XX Resolution 500–525 bands	Fig. 1F
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 knock-in region	Fig. 1B
	Evaluation of the − (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	N/A	
	Transgene-specific PCR	PCR for the (intact) transgene presence	Fig. 1B
Verification of the absence of random plasmid integration events	PCR	No integration detected	Supp. Fig. 1D
Parental and modified cell line genetic identity evidence	STR analysis	All 16 STR loci tested matched	Supp.File
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR product)	Heterozygous target insertion with correct border region	Supp. Fig. 1A-B
	PCR-based analyses	Heterozygous target integration	Fig. 1B
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	ND	N/A
Off-target nuclease activity analysis	PCR across top 3 predicted top likely off-target sites, whole genome/exome sequencing	No mutation detected	Supp. Fig. 1C
Specific pathogen-free status	Mycoplasma	Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza, Negative	Suppl. Fig. 1E
Multilineage differentiation potential	Directed differentiation	Successful differentiation towards three germ layers	Fig. 1G
List of recommended germ layer markers	Immunocytochemistry	Ectoderm: TUBB3, NESTIN Endoderm: SOX17, FOXA2 Mesoderm: SM22-α/TAGLN, SNAIL	Fig. 1G
Outcomes of gene editing experiment (OPTIONAL)	Brief description of the outcomes in terms of clones generated/establishment approach/screening outcomes	ND	N/A
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype − additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

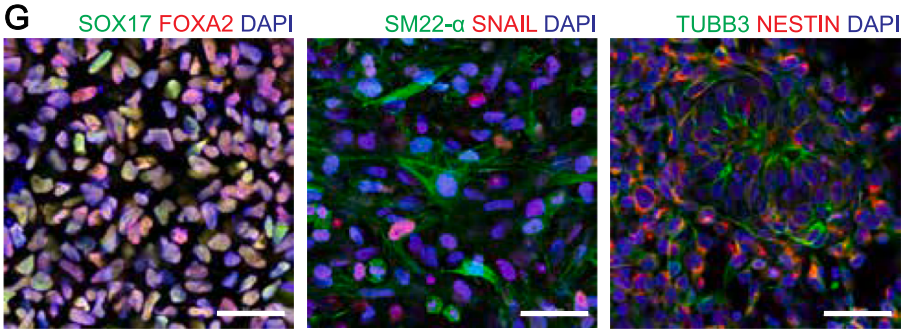


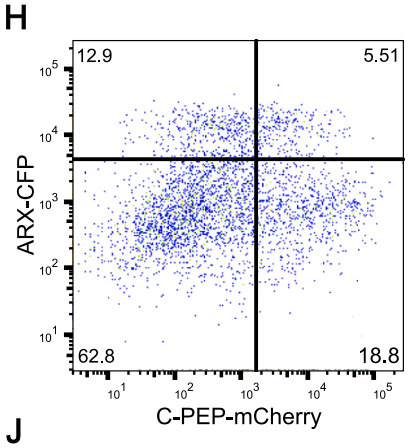


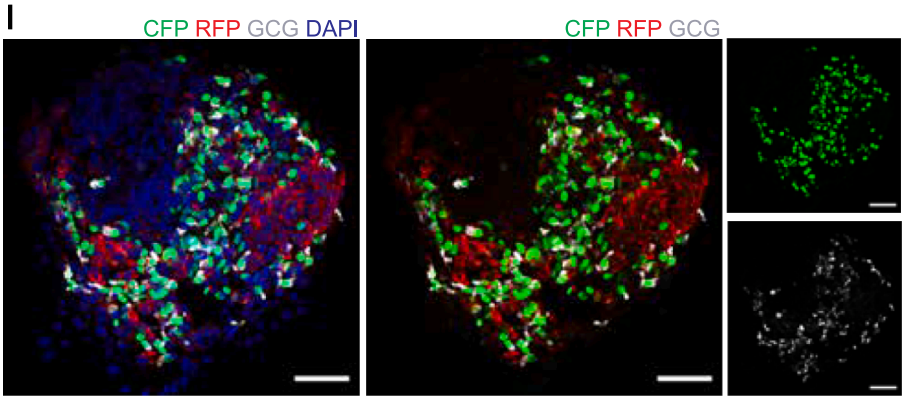


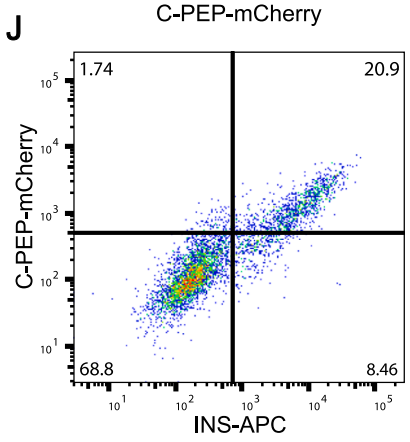












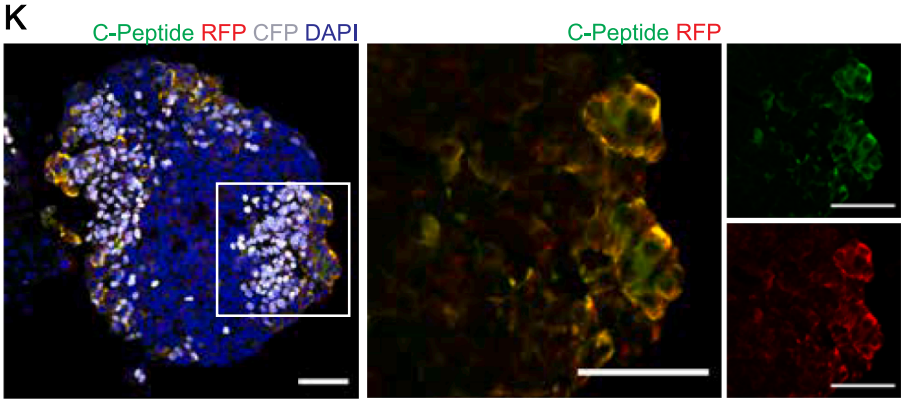


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Fig. 1. Generation and validation of ARX-T2A-H2B-CFP x C-PEP-mCherry-hiPSC (HMGUi001-A-54) line. (A) Schematic representation of mCherry integration in the Insulin gene, with the primer location for genotyping. (B) 5′ and 3′ PCR products of integration of targeting vector in the reporter double reporter hiPSC line and its parental line. (C) Phase contrast image of HMGUi001-A-54 (scale bar: 300 μm). Pluripotency of the hiPSC line is shown through immunostaining (D) and flow cytometry (E). (F) Karyotyping analysis showed normal female karyotype. (G) Three germ layer differentiation is confirmed through immunostaining of endoderm (SOX17, FOXA2), mesoderm (SM22-α, SNAIL), and ectoderm (TUBB3, NESTIN). (H) Differentiation efficiency of HMGUi001-A-54 towards pancreatic lineage. (I) Immunostaining of CFP, RFP, and Glucagon showing some overlap of CFP and Glucagon (scale bar: 50 μm). Specific integration of mCherry in the insulin gene was shown through flow cytometry with overlap with insulin staining (J) and immunostaining of C-Peptide (K, scale bar: 50 μm).

the area of CRISPR/Cas9 induced double-strand break (Supp. Fig. 1A-B). Additionally, mutation at three gene encoding sites with the highest sgRNA off-target score was not detected (Supp. Fig. 1C) and random integrations of the plasmid backbone were not observed (Supp. Fig. 1D).

The generated iPSC line showed normal colony morphology (Fig. 1C) and its pluripotency was confirmed through immunostaining and flow cytometry analysis (Fig. 1D, E). This iPSC line also showed normal karyotype (46, XX) and tested negative for mycoplasma (Fig. 1F and Supp. Fig. 1E). Moreover, the iPSC line was able to differentiate towards all three germ layers as shown by immunostaining for endoderm, mesoderm, and ectoderm specific markers (Fig. 1G). Importantly, the generated iPSC line showed efficient differentiation towards pancreatic lineage, as confirmed by its reporter expression (Fig. 1H). Its potency to differentiate towards stem cell-derived alpha cells was shown through glucagon staining (Fig. 1I). Correct insertion of mCherry fluorophore was verified through co-expression of insulin by flow cytometry and C-Peptide by immunostaining (Fig. 1J, K).

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat# and RRID**
Pluripotency Markers	Goat anti-OCT3/4	1:500	Santa Cruz Biotechnology Cat# sc-8628, RRID:AB_653551
	Rabbit anti-SOX2	1:500	Cell Signaling Technology Cat# 3579, RRID:AB_2195767
	Human anti-SSEA4-APC	1:11	Santa Cruz Biotechnology Cat# sc-21704, RRID:AB_628289
	Human anti-TRA-1–60-PE	1:11	Miltenyi Biotec Cat# 130–122-921, RRID:AB_2801969
	Isotype control, human IgG1, PE	1:11	Miltenyi Biotec Cat# 130–113-438, RRID:AB_2733893
	Isotype control, mouse IgG1k	1:11	BD Biosciences Cat# 557732, RRID:AB_396840
Differentiation markers	Goat anti-SOX17	1:400	R and D Systems Cat# AF1924, RRID:AB_355060
	Rabbit anti-FOXA2	1:500	Cell Signaling Technology Cat# 8186, RRID:AB_10891055
	Rabbit anti-SM22a	1:200	Abcam Cat# ab14106, RRID:AB_443021
	Goat anti-Snail	1:300	R and D Systems Cat# AF3639, RRID:AB_2191738
	Rabbit anti-TUBB3	1:1000	Abcam Cat# ab18207, RRID:AB_444319
	Mouse anti-Nestin	1:300	Abcam Cat# ab22035, RRID:AB_446723
	Guinea pig anti-Insulin	1:300	Bio-Rad Cat# 5330-0104G, RRID:AB_1605150
	Mouse anti-Glucagon	1:2000	Sigma-Aldrich Cat# G2654, RRID:AB_259852
	Alexa Fluor® 647 Mouse Anti-Insulin	1:40	BD Biosciences Cat# 565689, RRID:AB_2739331
Reporter marker	Rat anti-RFP	1:1000	ChromoTek Cat# 5f8, RRID:AB_2336064
	Chicken anti-GFP	1:800	Aves Labs Cat# GFP-1020, RRID:AB_10000240
Secondary antibodies	Donkey anti-rabbit IgG 488	1:800	Thermo Fisher Scientific Cat# A-21206, RRID:AB_2535792
	Donkey anti-goat IgG 555	1:800	Thermo Fisher Scientific Cat# A-21432, RRID:AB_2535853
	Donkey anti-mouse IgG 555	1:800	Thermo Fisher Scientific Cat# A-31570, RRID:AB_2536180
	Donkey anti-goat IgG 488	1:800	Thermo Fisher Scientific Cat# A-11055, RRID:AB_2534102
	Donkey anti-rabbit IgG 555	1:800	Thermo Fisher Scientific Cat# A-31572, RRID:AB_162543
	Donkey anti-chicken IgY	1:800	Jackson ImmunoResearch Labs Cat# 703–225-155, RRID:AB_2340370
	Donkey anti-rat IgG (H + L)-Cy3	1:800	Jackson ImmunoResearch Labs Cat# 712–165-153, RRID:AB_2340667
	Donkey anti-guineapig 647	1:800	Jackson ImmunoResearch Labs Cat# 706–605-148, RRID:AB_2340476
	Donkey anti-mouse 647	1:800	Jackson ImmunoResearch Labs Cat# 715–605-151, RRID:AB_2340863
Nuclear stain	DAPI	0.2 μg/mL	Sigma-Aldrich, Cat# D9542
Site-specific nuclease			
Nuclease information	Cas9	SpCas9-Venus Fusion	
Delivery method	Lipofection	Lipofectamine Stem Transfection Reagent (Fisher Scientific, Cat# STEM00003)	
Selection/ enrichment strategy	FACS	GFP sorting with FACS Aria™ III (BD Biosciences)	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Cloning targeting vector	5′ HA	GGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCACCTGGCCTTCAGCCTGCCTCAGC/ GTTATCCTCCTCGCCCTTGCTCACCATTGCCCTTTTTTGGAGGGACCCCTCCAGGGCCAAGGG	
	3′ HA	GCCACTCCACCGGCGGCATGGACGAGCTGAAGCGTGGCATTGTGGAACAATGCTGTAC/ GCTGGGTACCGGCCCCCCCTCGAGGTTCCCTGCTTCTCCTGGGCTGCAATC	
Genotyping	C-Peptide 5′ knock-in allele (1260 bp)	GTCAGGTGGGCTCAGGATTCCAG/ TGTTATCCTCCTCGCCCTTGCTC	
	C-Peptide 5′ wild type allele (1433 bp)	GTCAGGTGGGCTCAGGATTCCAG/ TCACAACAGTGCCGGGAAGTGGG	
	C-Peptide 3′ knock-in allele (1088 bp)	ACCTCCCACAACGAGGACTAC/TAGCAAAGGAAGCCAGCCAAGTCAC	
	C-Peptide 3′ wild type allele (1404 bp)	GGCAGCTCCATAGTCAG/TAGCAAAGGAAGCCAGCCAAG	
	RCAN3 (371 bp)	CTGTGAGCGGGAAACTATGC/GTCTTGGCCTCCCAAATTGC	
	PRSS27 (591 bp)	CTGCTATGGACCATGTCTTCAC/AGGGCTACTTTAGGAAGGAAGG	
	CYTH4 (473 bp)	CTCATGGAGCCGAGAGTCTAGC/GGCCACACTGTCTGTTGACC	
	Plasmid backbone (571 bp)	CATTGGAAACCGTTCTTCGGGGC/TGGCCCACTACGTGAACCATC	
Sequencing	C-Peptide sgRNA target site in the wild type allele	TCACAACAGTGCCGGGAAGTGGG	
	C-Peptide 3′ recombination border knock-in allele	TCACAACAGTGCCGGGAAGTGGG	
	C-Peptide 5′ recombination border knock-in allele	GCGGGCACTGTGTCTCCCTGACTG	
	RCAN3	CTGTGAGCGGGAAACTATGC	
	PRSS27	AGGGCTACTTTAGGAAGGAAGG	
	CYTH4	CTCATGGAGCCGAGAGTCTAGC	

4. Materials and methods

4.1. hiPSC culture

The generated line was cultured in feeder-free conditions on Geltrex (Life Technologies, Cat# A1413302) coated plates in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec, Cat# 130–104-368) under standard culture conditions (37 °C, 5 % CO_2). Cells were passaged at 80 % confluency using 0.5 mM EDTA (PanReac Applichem Cat# A4892,1000) and cultured with 10 μM ROCK inhibitor (Y-27632, Biotechne, Cat# 1254/10) for the first 24 h. The absence of mycoplasma was monitored periodically with MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Cat# LT07-710) according to the manufacturer's instructions.

4.2. Cloning of targeting constructs and transfection of hiPSCs

Targeting vectors (Cas9-Venus-sgRNA and targeting vector) were cloned as previously described (Siehler et al., 2021). HMGUi001-A-4 hiPSCs were seeded onto 6 well tissue culture plates (0.4 x 10^6 cells/well). Transfection was performed one day after seeding with 5 μl Lipofectamine Stem Transfection Reagent (Fisher Scientific, Cat# STEM00003), 1.25 μg targeting vector, 1.25 μg Cas9-Venus-sgRNA vector per well. Culture media was changed after overnight transfection. Venus-expressing transfected cells were sorted using FACS Aria™ III (BD Biosciences) 48 h after transfection, plated at low density and single cell-derived clones were picked and expanded according to previously published protocol (Yumlu et al., 2017). Genotypes of the clones were screened by PCR and verified by Sanger sequencing.

4.3. Pancreatic lineage differentiation

Differentiation towards pancreatic lineage was performed in 3D culture according to Velazco-Cruz et al., 2019. In brief, hiPSCs were seeded in 30 mL spinner flasks (Reprocell, Cat# ABBWVS03A-6) on a magnetic stirrer (60 rpm) with a seeding density of 0.8 x 10^6 cells/ml. Differentiation was started 3–4 days after seeding depending on aggregate size (approx. 200 μm in diameter). Aggregates were differentiated following the 6-stage protocol and analysed at Stage 6 day 14.

4.4. Three germ layer differentiation

hiPSC were differentiated towards the three-germ layer in a 2D monolayer using the StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, Cat# 130–115-660) following the manufacturer's directions.

4.5. STR analysis

STR Analysis was performed by Helmholtz Munich's Genomics Core Facility with AmpFLSTR™ Identifiler™ PCR Amplification Kit (Applied Biosystems).

4.6. Karyotyping

Karyotype analysis was performed in passage 40 by the Institute for Human Genetics of Technical University Munich as previously described by Wang et al., 2018.

4.7. Immunocytochemistry and flow cytometry analysis

Cell monolayers were fixed and stained as previously described by Wang et al., 2018. Aggregates from 3D differentiation were fixated, embedded, sectioned and stained according to Bastidas-Ponce et al., 2017. Flow cytometry analysis was performed with live and fixed single cells samples. hiPSCs were disscociated with Accutase® solution (Sigma-Aldrich, Cat# A6964) and stained with SSEA-4-FITC and TRA-1–60-PE conjugated antibodies and their respective isotype according to manufacturer's instructions. Differentiated stem cell-derived islets were dissociated using TrypLE™ Select (ThermoFisher Scientific, Cat# 12563011) and flow cytometry analysis were performed without further staining. To confirm the overlap of mCherry signal with Insulin, dissociated SC-islets were fixed with 4 % PFA and permeabilized with 0.1 % Triton-X with 0.1 M Glycine in PBS. Cells were then stained with conjugated insulin-APC antibody. Primary and secondary antibodies are listed in Table 2.

CRediT authorship contribution statement

Eunike Sawitning Ayu Setyono: Data curation, Formal analysis, Investigation, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nicole Katarina Rogers:** Validation, Writing – review & editing. **Anita Hofmann:** Investigation, Resources. **Heiko Lickert:** Conceptualization, Funding acquisition, Supervision. **Ingo Burtscher:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Heiko Lickert reports financial support was provided by European Union. Heiko Lickert reports financial support was provided by Federal Ministry of Education and Research Bonn Office. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2025.103685.

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