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Generation of a heterozygous C-peptide-mCherry reporter human iPSC line (HMGUi001-A-8)

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A B S T R A C T

The peptide hormone insulin produced by pancreatic β-cells undergoes post-transcriptional processing before secretion. In particular, C-peptide is cleaved from pro-insulin to generate mature insulin. Here, we introduce a C-peptide-mCherry human iPSC line (HMGUi001-A-8). The line was generated by CRISPR/Cas9 mediated heterozygous insertion of the mCherry sequence into exon 3 of the insulin locus. We demonstrate that the line is pluripotent and efficiently differentiates towards pancreatic β-like cells, which localize a red fluorescent C-peptide-mCherry fusion protein in insulin containing granules. Hence, the HMGUi001-A-8 line is a valuable resource to purify derived β-like cells and follow insulin-containing granules in real time.

1. Resource table

Unique stem cell line identifier	HMGUi001-A-8
Alternative name(s) of stem cell line	C-peptide-mCherry-hiPSC
Institution	Institute of Diabetes and Regeneration Research, Helmholtz Zentrum München, 85764 Neuherberg, Germany
Contact information of distributor	Heiko Lickert, heiko.lickert@helmholtz-muenchen.de
Type of cell line	iPSCs
Origin	Human, HMGUi001-A hiPSCs described in Wang et al., 2018
Additional origin info	Age: N/A Sex: Female Ethnicity: Caucasian
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	YES
Type of Modification	Heterozygous insertion of a fluorescent reporter
Associated disease	N/A
Gene/locus	Insulin gene (INS)/11p15.5
Method of modification	CRISPR/Cas9 mCherry
Name of transgene or resistance	
Inducible/constitutive system	N/A
Date archived/stock date	July 28, 2020
Cell line repository/bank	https://hpscreg.eu/cell-line/HMGUi001-A-8
Ethical approval	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.

2. Resource utility

The C-peptide-mCherry-hiPSC (HMGUi001-A-8) line is characterized by fusion of mCherry to the C-terminus of C-peptide, which is a

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield imaging	Normal morphology	Fig. 1C
Phenotype	Immunocytochemistry	Staining for OCT3/4 and SOX2	Fig. 1D
	Flow Cytometry	SSEA-4 and TRA-1-60 (98.0% double positive)	Fig. 1E
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 450–525 bands	Fig. 1G
Identity	Microsatellite PCR (mPCR) OR STR analysis	Not performed AmpF*ℓ*STR™Identifiler™ PCR Amplification Kit, 16 sites tested, all matched	submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous insertion of reporter cassette determined by PCR and confirmed by Sanger sequencing	Fig. 1B, Supplementary Fig. 1B
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza, Negative	Supplementary Fig. 1C
Differentiation potential	Directed differentiation	Three germ layer formation: FOXA2/SOX17: endoderm SM22-α/SNAIL: mesoderm TUBB3/PAX6: ectoderm	Fig. 1F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

byproduct generated during insulin synthesis and processing. Therefore, it allows detection and sorting of insulin positive β-like cells during pancreatic differentiation. Furthermore, intracellular trafficking of insulin containing vesicles can be monitored.

3. Resource details

Human induced pluripotent stem cells (hiPSCs) can be differentiated with a stepwise protocol towards insulin expressing pancreatic β-like cells, which have a broad range of applications in diabetes research. Among others, β-like cells could be used for cell replacement therapy for type 1 diabetes patients or for *in vitro* disease modeling. However, current differentiation protocols towards β-like cells result in heterogeneous cell populations. Besides functionally relevant insulin expressing β-like cells, other hormone secreting cell types as well as non-hormone producing progenitors and cells of other lineages are generated (Veres et al., 2019). C-peptide can be used to identify insulin positive β-like cells. It is cleaved from pro-insulin during post-transcriptional insulin processing and is then stored together with equimolar amounts of insulin in secretory granules.

The C-peptide-mCherry-hiPSC line (HMGUi001-A-8) was generated by heterozygous insertion of the mCherry sequence at the C-terminus of the C-peptide, which is encoded by exon 3 of the insulin locus (Table 1, Fig. 1A). The inserted mCherry sequence was followed by a repeat of the last two amino acids of C-peptide, as they are crucial for insulin processing by prohormone convertases 2 and 3. Importantly, the biological active insulin protein should not be affected by the genetic modifications (Supplementary Fig. 1A). Genome editing was performed by CRISPR/Cas9 targeting of the previously described iPSC line HMGUi001-A (Wang et al., 2018). Correct insertion was verified by PCR (Fig. 1B). Subsequent Sanger sequencing of the single guide RNA (sgRNA) targeted region revealed correct insertion in one allele and no unwanted mutation in the targeted allele or wild type allele (Supplementary Fig. 1B).

The generated line showed typical hiPSC colony formation (Fig. 1C). It was positive for the nuclear pluripotency markers SOX2 and OCT3/4 as well as the cell surface pluripotency markers SSEA-4 and TRA-1-60 (Fig. 1D and E). Furthermore, pluripotency was confirmed by successful differentiation towards all three germ layers, which was demonstrated by immunostaining for endoderm, mesoderm and ectoderm specific markers (Fig. 1F). Beyond that, the line revealed normal karyotype (46, XX) and was tested negative for mycoplasma (Fig. 1G, Supplementary Fig. 1C). No mutations at the three gene encoding sites with the highest sgRNA off target score were detected (Supplementary Fig. 1D).

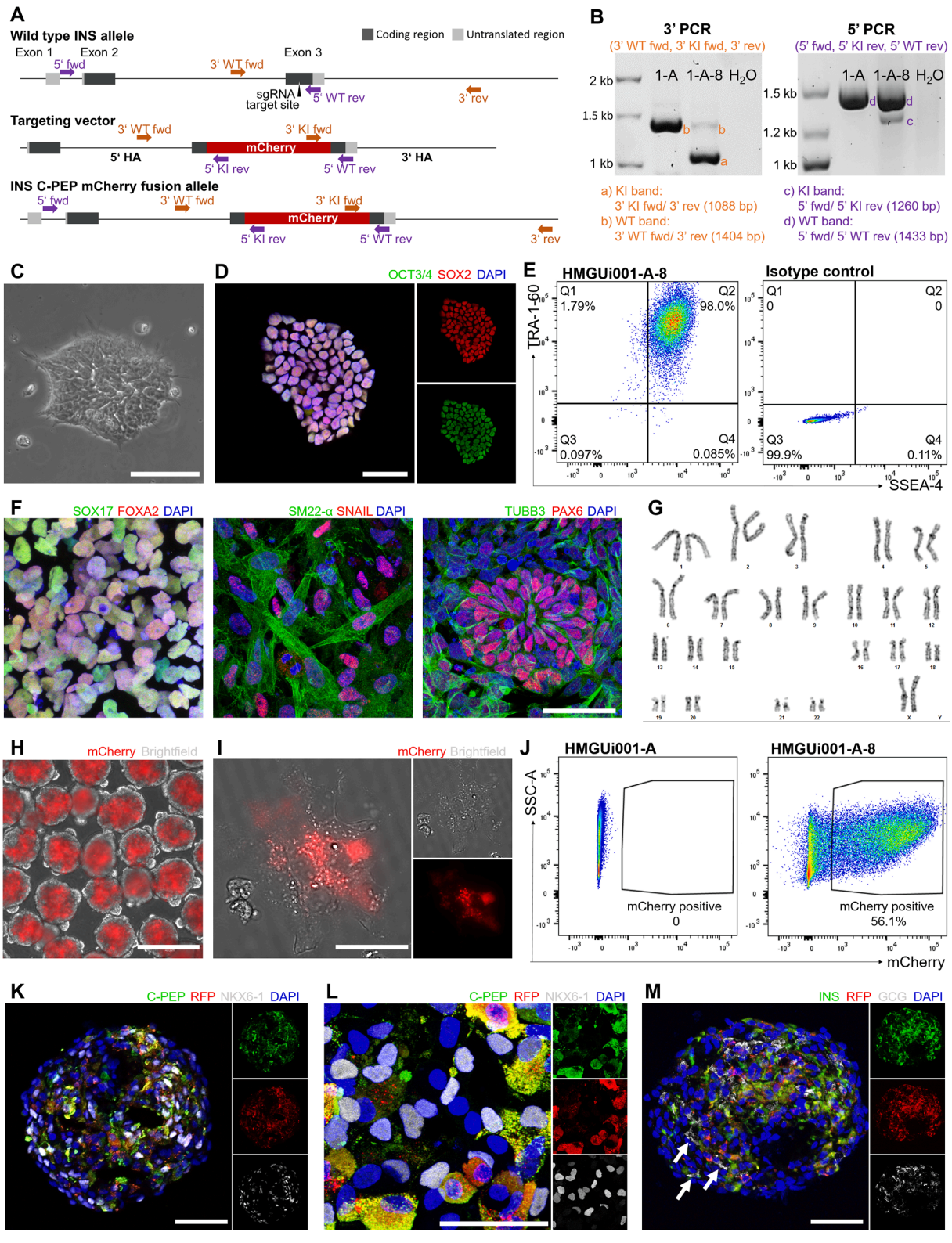
After differentiation of the line towards β-like cells, strong mCherry expression could be detected by live imaging as well as by flow cytometer analysis (Fig. 1H, I and J). Furthermore, immunostaining confirmed that mCherry (RFP) was co-expressed with both insulin and C-peptide (Fig. 1K, L & M). As expected, hormone negative and glucagon positive cells were negative for mCherry (Fig. 1M, arrows indicate glucagon mono-hormonal, RFP negative cells). Most mCherry/C-peptide positive cells expressed NKX6-1 indicating correct differentiation towards maturing β-like cells (Veres et al., 2019; Fig. 1K and L). C-peptide-mCherry intracellularly localized to insulin-containing granules, which transport insulin and C-peptide from the endoplasmic reticulum to the plasma membrane (Fig. 1I and L).

In summary, the HMGUi001-A-8 line allows for the monitoring of expression and intracellular localization of C-peptide during the differentiation towards pancreatic β-like cells. Beyond that, it can be used to generate a homogeneous insulin-positive cell population by fluorescence-based sorting of mCherry positive cells.

4. Materials and methods

4.1. HiPSC culture

HiPSCs were cultured on diluted Geltrex (Life Technologies, Cat# A1413302) coated tissue culture plates in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec, Cat# 130-104-368) under standard culture conditions (37°C, 5% CO_2). Medium was changed daily. Cells were



(caption on next page)

Fig. 1. Generation and quality controls of the heterozygous C-peptide-mCherry-hiPSC line (HMGUi001-A-8). **A** MCherry was inserted into the C-peptide (C-PEP) sequence in exon 3 of the insulin (INS) locus by Cas9 cutting upon sgRNA binding and homologous repair. The targeting vector containing the mCherry sequence flanked by a 5′ and 3′ homology arm (HA) served as template for the repair. The knock-in (KI) and wild type (WT) allele were identified by PCR for the 3′ recombination boarder (primers: orange arrows) and the 5′ recombination boarders (primers: purple arrows). **B** Knock in of mCherry in HMGUi001-A-8 (1-A-8) was verified by generation of the knock-in (KI) band in the 3′ PCR and 5′ PCR. DNA from HMGUi001-A (1-A) served as control. Both PCRs show the wild type (WT) PCR product indicating heterozygous insertion. PCR product corresponding primers are shown in orange (3′ PCR) and purple (5′ PCR). **C** Phase contrast image of an HMGUi001-A-8 colony two days after splitting. Scale bar indicates 100 μm. **D&E** Analysis of pluripotency by staining for pluripotency markers. HMUG001-A-8 is positive for the nuclear markers OCT3/4 and SOX2 (D) and the surface markers TRA-1-60 and SSEA-4 (E). Scale bar indicates 50 μm. **F** Directed differentiation of HMGUi001-A-8 *in vitro* towards the three germ layers. Their identity was confirmed by immunostaining (endoderm: SOX17, FOXA2; mesoderm: SM22-α, SNAIL; ectoderm: TUBB3, PAX6). Scale bar indicates 50 μm. **G** HMGUi001-A-8 is characterized by a normal female karyotype (46, XX). **H-J** Live detection of C-peptide-mCherry by fluorescence microscopy (H&I) and flow cytometry (J). HMGUi001-A-8 was differentiated towards β-like cells in 3D aggregate culture (H). Cells were dissociated for flow cytometry (J) or replated for 2D imaging (I). Scale bars indicate 400 μm (H) and 50 μm (I). **K-M** Aggregates differentiated towards β-like cells were fixated, sectioned and stained for C-peptide (C-PEP), insulin (INS), glucagon (GCG), mCherry (RFP) and NKX6-1. C-PEP signal overlaps with the RFP signal, but is absent from GCG monohormonal cells (arrows). Scale bars indicate 50 μm.

split or seeded for experiments with 0.5 mM EDTA (AppliChem, Cat#A4892) when reaching 70% confluency. After splitting or seeding, cells were cultured in 10 μM ROCK inhibitor (Y-27632, Santa Cruz Biotechnology, Cat# sc-281642A) for 24 h. Cells were regularly checked for Mycoplasma with the MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Cat# LT07-703) according to the manufacturer's instructions.

4.2. Cloning of targeting constructs

For the insertion of mCherry into exon 3 of the insulin locus, two vectors were generated. For the first vector, the Cas9-Venus-sgRNA vector, a sgRNA was cloned into the pU6-(*Bbs*I)-sgRNA-CAG-Cas9-Venus-bpA plasmid (Addgene plasmid #86986) by *Bbs*I digest of the vector and Gibson assembly. The sgRNA was specific to the targeting region in exon 3 and designed with the CRISPOR webtool (http://crispor.tefor.net). The second vector, the targeting vector, contained the template for the intended homology directed repair: the mCherry coding sequence obtained from a pCAG-T2A-H2B-Cherry plasmid flanked by a 1111 bp left homology arm (5′ HA) and a 901 bp right homology arm (3′ HA). Both homology arms were amplified by PCR using genomic DNA purified from HMGUi001-A hiPSCs. Thereby, silent mutations in the sgRNA binding site were introduced to avoid cutting of the correctly inserted sequences.

4.3. Transfection of hiPSCs

HMGUi001-A hiPSCs were seeded to 6 well tissue culture plates (0.4×10^6 cells per well). One day after seeding, cells were transfected with the transfection mix containing 5 μl Lipofectamine™ Stem Transfection Reagent (Fisher Scientific, Cat# STEM00003), 1.25 μg targeting vector and 1.25 μg Cas9-Venus-sgRNA vector per well. Transfected cells expressed Venus and were sorted by FACS. Sorted cells were plated at low density, single cell derived clones were picked and expanded according to Yumlu et al., 2017. The genotype of the clones was screened by PCR and verified by Sanger sequencing.

4.4. Differentiation towards β-like cells

HiPSCs were differentiated towards pancreatic β-like cells according to Velazco-Cruz et al., 2019. Briefly, 0.6×10^6 iPSCs per ml were aggregated in spinner flasks on a magnetic stirrer (60 rpm) and differentiation was started 72 h later. After reaching stage 5 of the protocol, cells were dissociated with Accutase solution (Sigma-Aldrich, Cat# A6964) and re-clustered in ultra-low attachment plates (Corning, Cat# 3471) on an orbital shaker (100 rpm). Cells were analyzed 14 days after re-clustering.

4.5. Three germ layer differentiation

Cells were differentiated in 2D monolayers to endodermal, mesodermal and ectodermal cells with the StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, Cat# 130-115-660) according to manufacturer's instructions.

4.6. Immunocytochemistry

Fixation and staining of cell monolayers were carried out according to Wang et al., 2018. Aggregates were fixated, embedded, sectioned and stained as described in Bastidas-Ponce et al., 2017. Primary and secondary antibodies are listed in Table 2.

4.7. Flow cytometer analysis

For pluripotency marker analysis, hiPSCs were dissociated with 0.5 mM EDTA. 1×10^6 cells were stained with the conjugated surface antibodies SSEA-4-FITC and TRA-1-60-PE and corresponding isotypes according to manufacturer's instructions (Table 2). For analysis of mCherry expression, differentiated aggregates were dissociated with Accutase solution and analyzed without further staining.

4.8. STR analysis

DNA was extracted from hiPSCs and analyzed with the AmpFℓ-STR™Identifiler™ PCR Amplification Kit (Applied Biosystems, Cat# 4322288) according to manufacturer's instructions.

4.9. Karyotyping

Karyotyping was performed by the Institute of Human Genetics, Technische Universität München as previously described by Wang et al., 2018.

Table 2
Reagents details.

Antibodies 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	1:400	Cell signaling Technology, Cat# 3579S, RRID: AB_2195767
	Human anti-SSEA4-FITC	1:11	Miltenyi Biotec, Cat# 130-098-371, RRID:AB_2653517
	Human anti-TRA-1-60-PE	1:11	Miltenyi Biotec, Cat# 130-100-347, RRID:AB_2654227
Isotype controls	REA Control (S)-PE-Vio615	1:11	Miltenyi Biotec, Cat# 130-107-146, RRID:AB_2661694
	REA Control (S)-FITC	1:11	Miltenyi Biotec, Cat# 130-104-610, RRID:AB_2661688
Germ layers	Rabbit anti-FOXA2	1:500	Cell signaling Technology, Cat# 8186S, RRID: AB_10891055
	Goat anti-SOX17	1:400	Neuromics, Cat# GT15094, RRID: AB_2195648
	Rabbit anti-SM22-α	1:100	Abcam, Cat# ab14106, RRID: AB_443021
	Rabbit anti-Tubulin beta III	1:1000	Abcam, Cat# ab18207, RRID: AB_444319
	Mouse anti-PAX6	1:100	DSHB Hybridoma, Cat# PAX6, RRID: AB_528427
	Goat anti-Snail	1:300	R&D Systems, Cat# AF3639, RRID: AB_2191738
Pancreatic progenitor Markers	Rabbit anti-NKX6.1	1:300	Arcis, Cat# NBP1-82553, RRID: AB_11023606
Hormones	Guinea pig anti-INS	1:400	Bio-Rad, Cat# 5330-0104G, RRID: AB_1605150
	Guinea pig anti-C-PEP	1:100	Abcam, Cat# ab30477, RRID: AB_726924
	Mouse anti-glucagon	1:600	Sigma, Cat# G2654-.2ML, RRID:AB_259852
mCherry	Rat anti-RFP	1:1000	Chromotek, Cat# 5F8, RRID: AB_2336064
Secondary antibodies	Donkey anti-rabbit Alexa Fluor 555 IgG	1:500	Invitrogen, Cat# A31572, RRID: AB_162543
	Donkey anti-goat Alexa Fluor 488 IgG	1:500	Invitrogen, Cat# A11055, RRID: AB_2534102
	Donkey anti-rabbit Alexa Fluor 488 IgG	1:500	Invitrogen, Cat# A21206, RRID: AB_2535792
	Donkey anti-mouse Alexa Fluor 555 IgG	1:500	Invitrogen, Cat# A31570, RRID: AB_2536180
	Donkey anit-goat Alexa Fluor 555 IgG	1:500	Invitrogen, Cat# A21432, RRID: AB_2535853
		1:500	

(*continued on next page*)

Table 2 (*continued*)

Antibodies used for immunocytochemistry/ flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
	Donkey anti-guinea pig 488 IgG		Dianova Cat# 706-545-148, RRID: AB_2340476
	Donkey anti-mouse 647 IgG	1:500	Dianova Cat# 715-605-151, RRID:AB_2340863
	Donkey anti-rabbit 647 IgG	1:500	Fisher Scientific, Cat# A31573, RRID:AB_ 2536183
	Donkey anti-rat Cy3 IgG	1:500	Dianova Cat# 712-165-153, RRID: AB_2340667

Primers		
	Target	**Forward/Reverse primer (5′-3′)**
Cloning targeting vector	5′ HA	GGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCACCTGGCCTTCAGCCTGCCTCAGC/ GTTATCCTCCTCGCCCTTGCTCACCATTGCCCTTTTTTGGAGGGACCCCTCCAGGGCCAAGGG
	3′ HA	GCCACTCCACCGGCGGCATGGACGAGCTGAAGCGTGGCATTGTGGAACAATGCTGTAC/ GCTGGGTACCGGCCCCCCCTCGAGGTTCCCTGCTTCTCCTGGGCTGCAATC
Oligo for cloning sgRNA		CACCGGGGAGGGGTCCCTGCAGAAGCG/AAACCGCTTCTGCAGGGACCCCTCCCC
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	CTGTGAGCGGGAAACTATGC/GTCTTGGCCTCCCAAATTGC
	PRSS27	CTGCTATGGACCATGTCTTCAC/AGGGCTACTTTAGGAAGGAAGG
	CYTH4	CTCATGGAGCCGAGAGTCTAGC/GGCCACACTGTCTGTTGACC
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

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.

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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.2020.102126.

References

Bastidas-Ponce, A., Roscioni, S.S., Burtscher, I., Bader, E., Sterr, M., Bakhti, M., Lickert, H., 2017. Foxa2 and Pdx1 cooperatively regulate postnatal maturation of pancreatic β-cells. Molecular Metabolism 6, 524–534.

Velazco-Cruz, L., Song, J., Maxwell, K.G., Goedegebuure, M.M., Augsornworawat, P., Hogrebe, N.J., Millman, J.R., 2019. Acquisition of dynamic function in human stem cell-derived β cells. Stem Cell Rep. 12 (2), 351–365.

Veres, A., Faust, A.L., Bushnell, H.L., Engquist, E.N., Kenty, J.H.R., Harb, G., Poh, Y., Sintov, E., Gürtler, M., Pagliuca, F.W., Peterson, Q.P., Melton, D.A., 2019. Charting cellular identity during human in vitro β-cell differentiation. Nature 569 (7756), 368–373.

Wang, X., Sterr, M., Burtscher, I., Chen, S., Hieronimus, A., Machicao, F., Staiger, H., Häring, H.-U., Lederer, G., Meitinger, T., Cernilogar, F.M., Schotta, G., Irmler, M., Beckers, J., Hrabě de Angelis, M., Ray, M., Wright, C.V.E., Bakthi, M., Lickert, H., 2018. Genome-wide analysis of PDX1 target genes in human pancreatic progenitors. Molecular Metabolism 9, 57–68.

Yumlu, S., Stumm, J., Bashir, S., Dreyer A.-K., Lisowski, P., Danner, E., Kühn, R., 2017. Gene editing and clonal isolation of human induced pluripotent stem cells using CRISPR/Cas9, Methods, 15, 121–122:29–44.