Stem Cell Research 45 (2020) 101797

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Generation of an INSULIN-H2B-Cherry reporter human iPSC line

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A B S T R A C T

Differentiating human induced pluripotent stem cells (hiPSCs) into insulin (INS)-producing β-like cells has potential for diabetes research and therapy. Here, we generated a heterozygous fluorescent hiPSC reporter, labeling INS-producing β-like cells. We used CRISPR/Cas9 technology to knock-in a T2A-H2B-Cherry cassette to replace the translational INS stop codon, enabling co-transcription and T2A-peptide mediated co-translational cleavage of INS-T2A and H2B-Cherry. The hiPSC-INS-T2A-H2B-Cherry reporter cells were pluripotent and showed multi-lineage differentiation potential. Cells expressing the β-cell specific hormone INS are identified by nuclear localized H2B-Cherry reporter upon pancreatic endocrine differentiation. Thus, the generated reporter hiPSCs enable live identification of INS hormone-producing β-like cells.

Resource table

Unique stem cell line identifier	HMGUi001-A-1
Alternative name(s) of stem cell line	hiPSC-INS-T2A-H2B-Cherry (+/-), AB001
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	Nucleofection
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
Name of transgene or resistance	Thosea asigna virus 2A (T2A)-histone 2B (H2B)-Cherry
Inducible/constitutive system	N/A
Date archived/stock date	February 2018
Cell line repository/bank	N/A
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 vote of the Ethics Committee of the Medical Faculty of the Eberhard Karls University, Tübingen (file numbers 629/2012BO2 and 130/2018BO2). The study design followed the principles of the Declaration of Helsinki. All study participants gave informed consent prior to entry into the study.

1. Resource utility

The generated heterozygous hiPSC-INS-T2A-H2B-Cherry reporter line enables live identification of INS-producing β-like cells during pancreatic differentiation. Expression of nuclear H2B-Cherry co-localizes with the β-cell-specific hormone INS and its cleavage peptide (C-peptide; C-PEP).

1.1. Resource details

Insulin (INS) is a hormone secreted by pancreatic β-cells in the islets of Langerhans. By releasing INS, β-cells promote glucose uptake in peripheral organs and consequently regulate constant blood glucose levels. Autoimmune destruction of β-cells results in INS deficiency in

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https://doi.org/10.1016/j.scr.2020.101797
Received 2 March 2020; Accepted 14 March 2020
Available online 22 April 2020

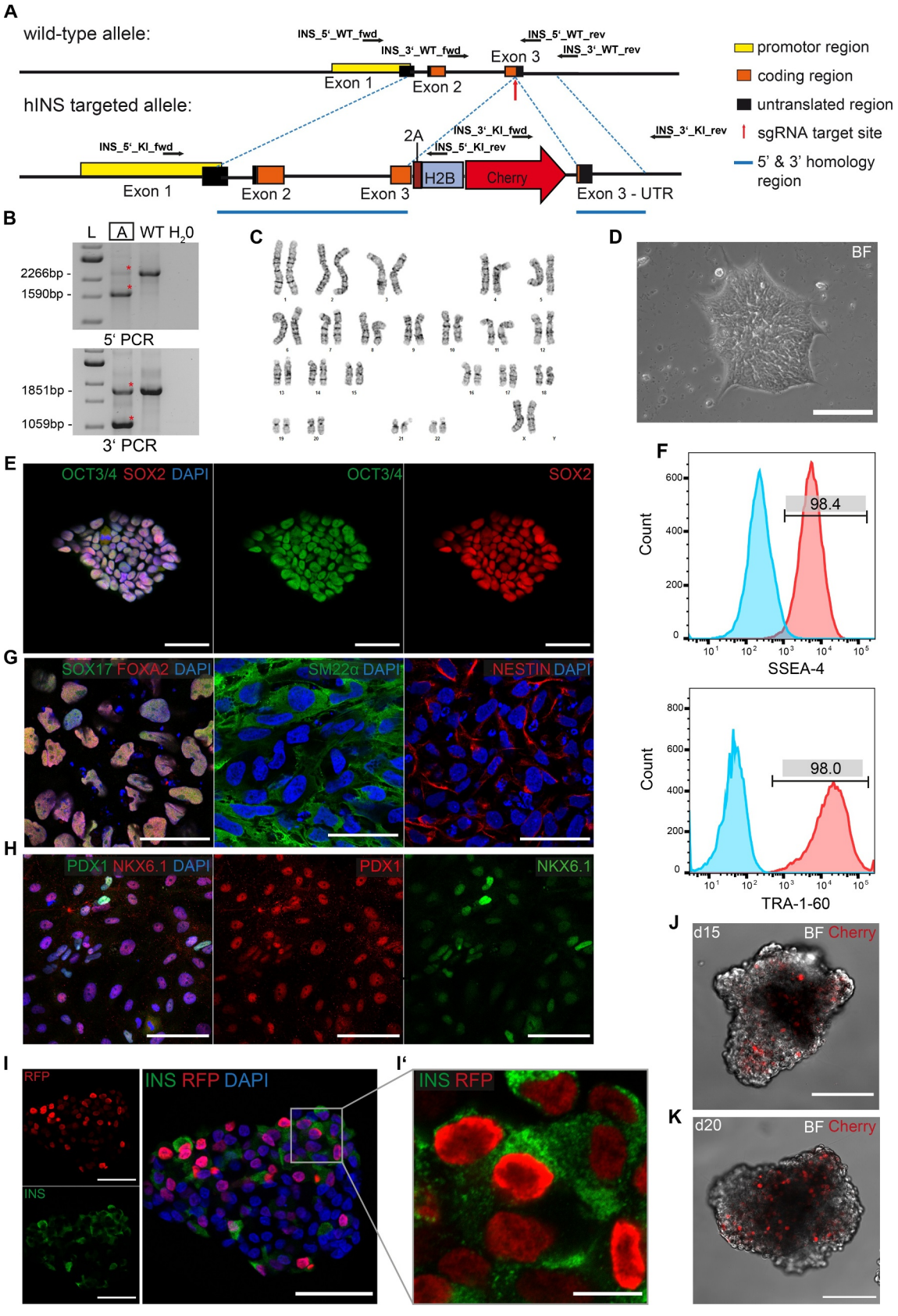

Fig. 1.

patients with type 1 diabetes (T1D). To compensate for the loss of endogenous β-cells in T1D, generating INS producing β-like cells from hiPSCs *in vitro* is a promising approach for cell-replacement therapy. Monitoring the expression of INS throughout the differentiation is a powerful tool to improve differentiation of functionally relevant β-like cells. Here, we generated a hiPSC-INS-T2A-H2B-Cherry reporter to

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal morphology	Fig. 1 panel D Scale bar: 100μm
Phenotype	Immunocytochemistry	Staining for OCT3/4 and SOX2	Fig. 1 panel E Scale bars: 50μm
	Flow Cytometry	SSEA-4 (98.4%) TRA-1-60 (98.0%)	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 450–525 bands	Fig. 1 panel C
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, three sgRNA putative off-target sites analyzed by Sanger sequencing	Fig. 1 panel B, Supplementary Fig. S1 panel A, B, C Supplementary Fig. S1 panel E
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Biochemical luminescence MycoAlert™ Plus Mycoplasma Detection Kit, Lonza, Negative	Supplementary Fig. S1 panel D
Differentiation potential	Directed differentiation	Three germ layer formation: FOXA2/SOX17: endoderm; SM22α: mesoderm; NESTIN: ectoderm	Fig. 1 panel G Scale bars: 50μm
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis Css	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

monitor live β-like cell formation during pancreatic endocrine differentiation *in vitro*. We targeted exon 3 of the *INS* gene of the previously described hiPSCs HMGUi001-A (Wang et al., 2018) to generate a C-terminal fusion of INS with T2A-H2B-Cherry, using CRISPR/Cas9 genome editing (Fig. 1A). The T2A self-cleaving sequence was used to generate a bi-cistronic reporter cassette enabling equimolar expression of H2B-Cherry and INS-T2A and thus avoiding loss of one copy of the endogenous *INS* gene. The heterozygous insertion of the targeting vector in clone A was confirmed by PCR analysis (Fig. 1B). The integrity of the non-targeted allele in clone A was confirmed by Sanger sequencing of the single guide RNA (sgRNA) target site in the non-targeted allele (Fig. S1A). Correct integration of the targeting vector was validated by sequencing the 5′ and 3′ recombination borders of the knock-in (KI) allele (Fig. S1B and S1C) (Tables 1 and 2).

The generated reporter cells had a normal karyotype (Fig. 1C) and formed colonies with normal hiPSC morphology (Fig. 1D). Short tandem repeat (STR) analysis confirmed derivation from the parental HMGUi001-A hiPSC line and the generated hiPSC line was not contaminated with mycoplasma (Fig. S1D).

We performed sgRNA off-target analysis on coding regions with the highest off-target scores. The analysis revealed no off-target effects (Fig. S1E). The differentiation ability of the newly generated reporter cell line was assessed by directed *in vitro* differentiations. INS-H2B-Cherry hiPSCs expressed markers for endoderm (FOXA2/SOX17), mesoderm (smooth muscle 22 alpha (SM22α)) and ectoderm (NESTIN), and thus markers of all three germ layers (Fig. 1G).

Stage-wise pancreatic endocrine differentiation according to Rezania et al., 2014 was performed to test β–like cell differentiation ability. INS-H2B-Cherry hiPSCs showed expression of the pancreatic progenitor transcription factors PDX1 and NKX6.1 after 10 days of differentiation (Fig. 1H). INS-H2B-Cherry hiPSCs showed a bright nuclear H2B-Cherry signal in live imaging at day 15 (Fig. 1J) and at day 20 (Fig. 1K) during differentiation. Further characterization by immunocytochemistry showed co-localization of nuclear H2B-Cherry signal (stained with anti-RFP antibody (AB)) with cytoplasmic INS (Fig. 1I and I') and cytoplasmic C-PEP staining (Fig. S1F and S1F') after 20 days of differentiation.

2. Materials and methods

2.1. CRISPR/Cas9 genome editing

The INS-T2A-H2B-Cherry targeting vector was cloned by traditional cloning. The targeting vector contained a 1338 bp 5′ homology region (HR), T2A-H2B-Cherry coding sequences cloned from a pCAG-T2A-H2B-Cherry plasmid and a 754 bp 3′ HR. HRs were amplified by PCR using genomic DNA extracted from HMGUi001-A hiPSCs. For CRISPR/Cas9 mediated targeting we designed a sgRNA binding upstream of the INS stop codon sequence (using CRISPOR website (http://crispor.tefor.net). The specific sgRNA was cloned into the BbsI site of the pU6-(BbsI)-sgRNA-CAG-Cas9-Venus-bpA plasmid (Addgene plasmid #86986). Approximately 2 × 10^5 HMGUi001-A hiPSCs were transfected with Lipofectamine™ Stem Transfection Reagent (Fisher Scientific, Cat# STEM00003) to deliver 1.25 μg sgRNA/Cas9-Venus expressing plasmid and 1.25 μg targeting vector. Cells expressing sgRNA/Cas9-Venus were selected by sorting highly GFP expressing cells using flow cytometry. Cells were seeded at low density to obtain colonies derived from single cells. Single colonies were picked and expanded. Detailed transfection, sorting and expansion conditions are described in Yumlu et al., 2017. Correct insertion of the targeting construct was validated by PCR and Sanger sequencing.

2.2. Cell culture

Cells were cultured on Geltrex (Life Technologies, Cat# A1413302) coated plates using StemMACS™ iPS-Brew XF medium (Miltenyi Biotec, Cat# 130-104-368). The culture medium was replaced daily. Cells were passaged every three to four days using 0.5 mM EDTA (AppliChem, Cat# A4892) in PBS. 10 μM ROCK Inhibitor (Y-27632, Santa Cruz Biotechnology, Cat# sc-281642A) was added for 24 h after splitting. Incubation was performed at 37 °C, 5% CO_2 and 21% O_2.

2.3. Three germ layer differentiation

Directed three germ layer differentiation was performed using StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, Cat# 130-115-660) according to manufacturer's instructions. Cells were stained for expression of endoderm, mesoderm and ectoderm markers.

2.4. Pancreatic differentiation

Differentiation towards pancreatic β-like cells was performed according to Rezania et al., 2014. 1.25 μM IWP-2 (Tocris-Bioscience, Cat# 3533/10) was added during stage 2 (S2) of differentiation. After 10 days of differentiation, cells were collected and re-seeded to form 3D aggregates (~ 600 cells/aggregate) by using AggreWell™ 400 plates

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, used for FACS	Miltenyi Biotec Cat# 130-098-371, RRID:AB_2653517
	Human anti-TRA-1-60-PE	1:11, used for FACS	Miltenyi Biotec Cat# 130-100-347, RRID:AB_2654227
Isotype controls	REA Control (*S*)-PE-Vio615	1:11, used for FACS	Miltenyi Biotec Cat# 130-107—146, RRID:AB_2661694
	REA Control (*S*)-FITC		1:11, used for FACS Miltenyi Biotec Cat# 130-104-610, RRID:AB_2661688
Germ layers	Rabbit anti-FOXA2	1:1000	Cell signaling Technology Cat# 8186S, RRID:AB_10891055
	Goat anti-SOX17	1:1000	Neuromics Cat# GT15094, RRID:AB_2195648
	Rabbit anti-SM22α	1:100	Abcam Cat# ab14106, RRID:AB_443021
	Mouse anti-NESTIN	1:300	Abcam Cat# ab22035, RRID:AB_446723
Pancreatic progenitor Markers	Goat anti-PDX1	1:500	R&D Systems Cat# AF2419, RRID:AB_355,257
	Rabbit anti-NKX6.1	1:300	Arcis Cat# NBP1-82553, RRID:AB_11023606
Hormone Markers	Rabbit anti-INS	1:400	Cell signaling Technology, Cat# 3014, RRID:AB_2126503
	Guinea pig anti-C-PEP	1:100	Abcam Cat# ab30477, RRID:AB_726924
Cherry Marker	Rat anti-RFP	1:1000	Chromotek Cat# 5F8, RRID:AB_2336064
Secondary antibodies	Donkey anti-rabbit Alexa Fluor 555 IgG	1:800	Invitrogen Cat# A31572, RRID:AB_162543
	Donkey anti-goat Alexa Fluor 488 IgG	1:800	Invitrogen Cat# A11055, RRID: AB_2534102
	Donkey anti-rabbit Alexa Fluor 488 IgG	1:800	Invitrogen Cat# A21206, RRID:AB_2535792
	Donkey anti-mouse Alexa Fluor 555 IgG	1:800	Invitrogen Cat# A31570, RRID:AB_2536180
	Donkey anti-guinea pig DyLight 649	1:800	Dianova Cat# 706-605-148, RRID:AB_2340476
	Donkey anti-rat Cy3 IgG (*H* + *L*)	1:800	Dianova Cat# 712-165-153, RRID:AB_2,40667
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Cloning	T2A-H2B-Cherry/1238bp	ACTAGTTTACTTGTACAGCTCGTCCATGCCG/CCTGGGCAACGTGCTGGTTATTG	
	5′ homology arm/1338bp	GCGGCCGCAAGAGGCCATCAAGCAGGTCTGTTC/ACGCGTCGTTGCAGTAGTTCTCCAGCTGGTAGAG	
	3′ homology arm/754bp	GTCGACGCTGGAGAACTACTGCAACTAGAC/GGTACC AGCTCATGGTGCCATCTGAC	
Oligo for cloning sgRNA	Guide 4A	CACCGGGCTGGTAGAGGGAGCAGATGC/AAACGCATCTGCTCCCTCTACCAGCCC	
Genotyping	INS 5‘ knock-in (KI) allele/1590bp	GTGCTGACGACCAAGGAGATCTTC/CTCAACGTCGCCGCATGTTAG	
	INS 5‘ wild-type (WT) allele/2266bp	GTGCTGACGACCAAGGAGATCTTC/CAGCTCATGGTGCCATCTGAC	
	INS 3‘ knock-in (KI) allele/1059bp	AACAGTACGAACGCGCCGAG/AGCCAAGCAGCCCTGCTTAC	
	INS 3‘ wild-type (WT) allele/1851bp	GTGAGCCAACTGCCCATTGC/AGCCAAGCAGCCCTGCTTAC	
Sequencing	Amplification of the non-targeted allele	GTGCTGACGACCAAGGAGATCTTC/TCACAACAGTGCCGGGAAGTGGG	
	Seq sgRNA target site in the non-targeted allele	TCACAACAGTGCCGGGAAGTGGG	
	INS 5‘ KI allele/1590bp	GTGCTGACGACCAAGGAGATCTTC/CTCAACGTCGCCGCATGTTAG	
	Seq 5′ recombination border	GTGCTGACGACCAAGGAGATCTTC	
	INS 3‘ KI allele/1073bp	AACAGTACGAACGCGCCGAG/TAGCAAAGGAAGCCAGCCAAG	
	Seq 3′ recombination border	AGCCAAGCAGCCCTGCTTAC	
Off-target analysis	CAAP1/587bp	CAGGTTGGCACTGCTATTG/AGGTCATGCCACTGCACAC	
	COA1/622bp	GCTTTGACCACAGCACAAAC/ACCAGATCAGACCCTCAGTAAC	
	RADIL/584bp	GAGGAGTTCTCCCTGAAG/GTCTCATAGCACCAGGAC	

(Stem Cell Technologies, Cat# 34415). Cells were fixed after three days (S1), ten days (S4) and 20 days (S6) of differentiation. Cells were stained for endoderm markers (FOXA2/SOX17), pancreatic progenitor markers (PDX1/NKX6.1) and hormones (INS/C-PEP). For labeling H2B-Cherry expressing cells, RFP AB was used (Table 2).

2.5. Immunocytochemistry

Fixation and staining of adherent cells were performed as described in Wang et al., 2018. Embedding, sectioning and staining of 3D cell aggregates were performed according to Bastidas-Ponce et al., 2017. Detailed information about 1° ABs and 2° ABs is listed in Table 2.

2.6. Flow cytometry

Flow cytometry was used to quantify cellular expression of pluripotency markers. Cells were washed twice with PBS and detached with TrypLE™ Select Enzyme (Thermo Fisher Scientific, Cat# 12563011) for 3–4 min. Approximately 1×10^6 cells were stained with conjugated surface ABs SSEA-4-FITC and TRA-1-60-PE according to manufacturer's instructions. Isotype controls were included. Detailed information about surface ABs and isotype controls is listed in Table 2.

2.7. STR analysis and karyotyping

STR analysis was performed using the AmpFℓSTR™Identifiler™ PCR Amplification Kit (appliedbiosystems, Cat# 4322288) according to manufacturer's instructions. Karyotyping was performed as described before (Wang et al., 2018). Karyotyping and STR analysis were performed by the Institute of Human Genetics, Technische Universität München and Helmholtz Zentrum München, Munich, Germany.

Declaration of Competing Interest

All authors declare no conflicts of interest.

Acknowledgements

We thank G. Lederer, G. Eckstein and T. Meitinger for the karyotyping and for the STR analysis of our cells. We thank X. Wang for generating the parental hiPSC line. We are grateful to R. Kühn for discussion and advice. The project was funded by the German Federal Ministry of Education and Research(project number 01EK1607A).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101797.

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