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Lab Resource: Stem Cell Line

Generation and characterization of a human iPSC cell line expressing inducible Cas9 in the "safe harbor" AAVS1 locus

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ABSTRACT

We report the generation-characterization of a fetal liver (FL) B-cell progenitor (BCP)-derived human induced pluripotent stem cell (hiPSC) line CRISPR/Cas9-edited to carry/express a single copy of doxycycline-inducible Cas9 gene in the "safe locus" AAVS1 (iCas9-FL-BCP-hiPSC). Gene-edited iPSCs remained pluripotent after CRISPR/Cas9 genome-edition. Correct genomic integration of a unique copy of Cas9 was confirmed by PCR and Southern blot. Cas9 was robustly and specifically expressed on doxycycline exposure. T7-endonuclease assay demonstrated that iCas9 induces robust gene-edition when gRNAs against hematopoietic transcription factors were tested. This iCas9-FL-BCP-hiPSC will facilitate gene-editing approaches for studies on developmental biology, drug screening and disease modeling.

Resource Table

Unique stem cell line identifier	JCLRIi001-A-1
Alternative name of stem cell line	iCas9-FL-BCP-hiPSC
Institution	Josep Carreras Leukemia Research Institute
Contact information of distributor	Julio Castaño, jcastano@carrerasresearch.org
Type of cell line	iPSC
Origin	human
Additional origin info	Age: 19–22 weeks of human fetal development Sex: XX
Cell source	Fetal liver B-cell progenitors
Method of reprogramming	Non-integrative (Sendai virus)
Associated disease	Non applicable
Gene/locus	Cas9 inserted in AAVS1 locus
Method of modification	CRISPR-Cas9
Gene correction	NO
Name of transgene or resistance	Cas9
Inducible/constitutive system	Doxycycline inducible system
Date archived/stock date	December 2016
Cell line repository/bank	
Ethical approval	Patient's informed consent obtained. Institutional Review Board approval obtained (CMRB-CEIC-26/2013)

Resource utility

This iCas9-FL-BCP-iPSC constitutes a unique tool facilitating the screening on multiple sgRNAs (and libraries) for the generation of locus-specific genetic-edited (knock-in, knock-out, codon substitution, structural rearrangements, etc.) hiPSC for developmental biology, compound screening and disease modeling.

Resource details

Fresh fetal liver (FL) was collected from developing human embryos aborted at 19–22 weeks of pregnancy. Human tissue was provided by The Vrelinghuis abortion clinic (Utrecht, The Netherlands) upon signed

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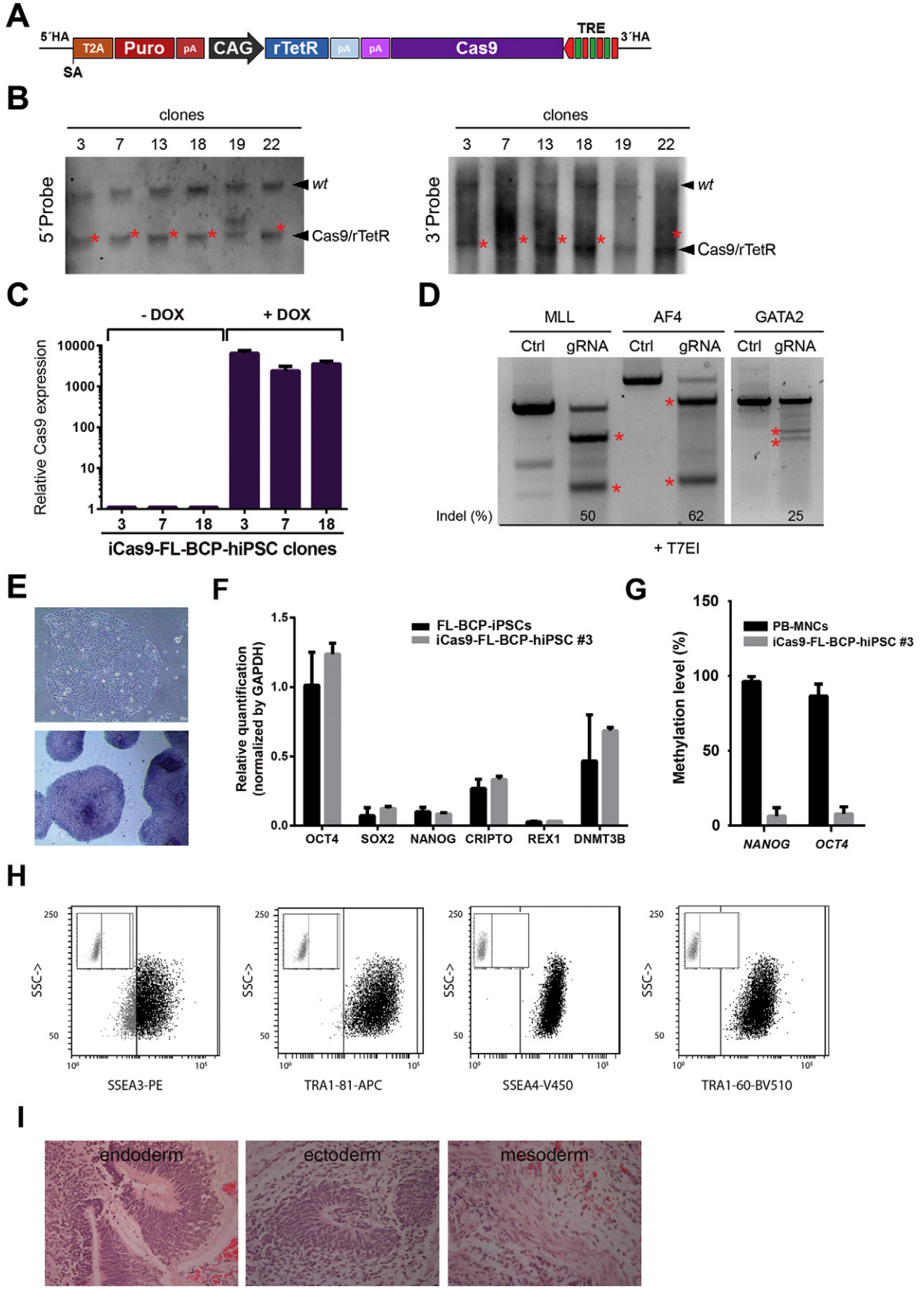

Fig. 1. CRISPR/Cas9-mediated generation of iCas9-FL-BCP-hiPSCs by gene targeting at the AAVS1 locus.

informed consent and approval by our local Ethics and Biozahard Board Committees (CMRB-CEIC-26/2013) through a formal collaboration with the Erasmus-Medical Centre, Rotterdam, The Netherlands). Mononuclear cells (MNCs) were isolated using Ficoll-Hypaque and CD34 + CD19+ B-cell progenitors (BCP) were FACS-purified and reprogrammed by infection with non-integrative tetracistronic SeV vectors encoding the transcription factors OCT4, SOX2, KLF4, and MYC (OSKM) (Muñoz-López et al., 2016a; Muñoz-López et al., 2016b). Resulting FL CD34 + CD19 + −iPSC lines (FL-BCP-hiPSC) were established as previously reported (Muñoz-López et al., 2016a; Muñoz-López et al., 2016b; Bueno et al., 2016). This FL-BCP-hiPSC was genome-edited to harbor the Cas9 coding sequence controlled by a doxycycline-inducible cassette in the genomic "safe harbor" AAVS1 (iCas9-FL-BCP-hiPSC). A single cassette containing both the rTetR activator under CAG promoter and the Tetracycline Response Element (TRE) promoter driving the expression of Cas9, was inserted in the AAVS1 locus by homologous recombination using the Cas9 nuclease and a guide RNA (gRNA) sequence (Mali et al., 2013) against intron 1 of AAVS1 locus (Fig. 1 panel A). The Fig. 1 panel A shows a schematic representation of the donor vector used for insertion of the iCas9 cassette into the AAVS1 locus (HA, homology arm; puro, puromycin; SA, splice acceptor; T2A, self-cleaving 2A peptide; CAG, CMV early enhancer/chicken β actin promoter. rTetR, reverse Tet repressor; pA, poly A signal; TRE, Tet response element). Correct genomic integration of a unique copy of Cas9 was confirmed by both genomic PCR (not shown) and Southern blot analysis (Fig. 1 panel B) in several iPSC clones, using a 5′-internal probe (left panel) and a 3′-external probe (right panel). Red asterisks indicate clones with the desired targeted insertions of the iCas9. Three iCas9-FL-BCP-hiPSC clones were induced for 72 h with 2 μg/ml of doxycycline and analyzed for Cas9 expression by qPCR (Fig. 1 panel C), showing a robust, non-leaky expression of Cas9. To functionally validate the Cas9 expression, iCas9-FL-BCP-hiPSCs were nucleofected with different gRNAs against three hematopoietic transcription factors (*MLL*, *GATA2* and *AF4*) in presence/absence of doxycycline. The T7 endonuclease I assay confirmed a high percentage (25%–62%) of cleavage (Fig. 1 panel D). Red asterisks depict the expected T7EI-specific fragments used to quantify indel frequency. The in silico-predicted (crispr.mit.edu) top off-targets of AAVS1 gRNA (*RNF4*, *RHOT2*, *FAIM2*, *RPL8*, *BTNL8*, *MYBL2*) were sequence-verified in iCas9-FL-BCP-hiPSCs and they were consistently found unaltered, demonstrating the high specificity of the approach used (data not shown).

Importantly, iPSCs remained pluripotent after CRISPR/Cas9 gene editing. iCas9-FL-BCP-hiPSCs retained hESC-like morphology and expressed the pluripotency markers alkaline phosphatase (AP) (Fig. 1 panel E), *OCT4*, *NANOG*, *SOX2*, *REX1*, *CRIPTO*, and *DNMT3B* (Fig. 1 panel F). Endogenous expression of *NANOG* and *OCT4* was accompanied by the extensive loss of CpG methylation in their promoters (Fig. 1 panel G). By flow cytometry, gene-edited iPSCs consistently expressed SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 1 panel H). In vivo, the differentiation capacity was confirmed by teratoma formation in NSG mice comprising tissue representing all three germ layers (Fig. 1 panel I).

Materials and methods

iPSC generation, maintenance and characterization

iPSCs were generated using OKSM polycistronic SeV vector (Muñoz-López et al., 2016a; Muñoz-López et al., 2016b) and were fully characterized before and after gene edition as previously described (Bueno et al., 2016).

Promoter demethylation

Bisulfite pyrosequencing of *OCT4* and *NANOG* promoters was done as described (Bueno et al., 2016).

Immunophenotyping

Antibodies used to check by flow cytometry the pluripotency-associated markers are detailed in Table 2.

CRISPR/Cas9-edition of hiPSCs expressing a doxycycline-inducible Cas9

The CAG-rTetR cassette was PCR amplified from AAVS1-Neo-M2rtTA (Addgene #60843) with primers containing restriction sites for *SalI* and *ClaI*. After amplification and enzyme-digestion, the CAG-rTetR cassette was cloned in the Puro-Cas9 donor vector (Addgene #58409). Finally, a gBlock fragment (IDT Technologies) designed with two opposite poly-A sequences was cloned using *MluI* and *ClaI* enzymes. A gRNA sequence targeting the *AAVS1* intron 1 (5′-GGGGCCACTAGGGACAGGAT-3′) was in vitro transcribed (IVT). To edit the iPSCs, 200.000 cells were electroporated with 100 pmol Cas9 nuclease (IDT - Integrated DNA Technologies), 120 pmol the IVT-gRNA against *AAVS1* intron 1, and 5 μg of linearized donor vector. Electroporation was performed using Neon Transfection System (ThermoFisher) at 1400 V, 5 ms and 3 pulses in a 100 μl tip. Cells were then selected with 1 μg/ml puromycin.

Southern blot

Genomic DNA from each cell line was isolated with Maxwell® RSC Cultured Cells DNA Kit (Promega). 6 μg of DNA from each clone was digested with *SphI* (for 5′ probe) or *BglII* (for 3′ probe) (New England Biolabs), separated on a 1% agarose gel and transferred to a nylon membrane (RPN303B, Amersham). Membranes were hybridized with DIG-dUTP labeled probes. Probes were detected by an AP-conjugated DIG-Antibody (Roche Diagnostics) using CDP-Star (Sigma-Aldrich) as

Table 1
Summary of quality control testing and results for iCas9-FL-BCP-hiPSC.

Classification	Test	Result	Data
Morphology	Photography	hESC-like morphology	Fig. 1 panel E top
	AP staining	Positive	Fig. 1 panel E bottom
Phenotype	qPCR	Expression of pluripotency markers: *OCT4*, *NANOG*, *SOX2*, *CRIPTO*, *REX*, *DNMT3B*	Fig. 1 panel F
	Promoter demethylation	loss of CpG methylation in *OCT4* and *NANOG* promoters	Fig. 1 panel G
	Flow cytometry	SSEA-3 (76%), SSEA-4 (100%), TRA-1-60 (100%) and TRA-1-81 (99%)	Fig. 1 panel H
Genotype	Karyotype	47 (XX) + 20 Resolution: 400-band level	Fig. 1S panel C
Identity	VDJH (BCR) rearrangement	Incomplete VDJH rearrangement (progenitor B cell)	Fig. 1S panel A
Mutation analysis	Southern blot	One specific insertion at *AAVS1* locus	Fig. 1 panel B
Microbiology and virology	Mycoplasma	Mycoplasma tested by PCR: negative	Fig. 1S panel B
Differentiation potential	Teratoma formation	Representation of all three germ layers	Fig. 1 panel I
Donor screening	N/A		
Genotype additional info	N/A		

Table 2
Antibodies and primers used in this study.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
SSEA-3-PE	Rat anti-SSEA-3	1:100	BD Bioscience Cat#560237, RRID:AB_1645542
SSEA-4-v450	Mouse anti-SSEA-4	1:100	BD Bioscience Cat#561156, RRID:AB_10896140
TRA-1-60-BV510	Mouse anti-TRA-1-60	1:100	BD Bioscience Cat#563188, RRID:AB_2637036
TRA-1-81-AlexaFlour647®	Mouse anti-TRA-1-81	1:100	BD Bioscience Cat#560793, RRID:AB_10550550
Primers			
	Target	Forward/Reverse primer (5′–3′)	
Genomic PCR	5′ junction	CTGCCGTCTCTCTCCTGAGT/GTGGGCTTGTACTCGGTCAT	
	3′ junction	GGCGATCTGACGGTTCACTAAAC/GAATCCACCCAAAAGGCAGC	
Southern blot	5′ probe	AGGTTCCGTCTTCCTCCACT/GTCCAGGCAAAGAAAGCAAG	
	3′ probe	ACAGGTACCATGTGGGGTTC/CTTGCCTCACCTGGCGATAT	
T7 assay	*MLL*	CAGCACTCTCTCCAATGGCA/TAAGCCTCCCATCTCCCACA	
	AF4	GGGGAAAAAAAAACATTTCGGCGACATG/CTACCATTTCCCTCATTCCAATTCACTCC	
	GATA2	CGTGTCGCTGGGATCAAG/TCCCCAAAGAAAGCCAGAAAC	
RNA in vitro transcription	*AAVS1* IVT	GAAATTAATACGACTCACTATAGGGGGCCACTAGGGACAGGATGTTTTAGAGCTAGAAA/AAAAGCACCGACTCGGTGCC	
	MLL IVT	GAAATTAATACGACTCACTATAGTTAGCAGGTGGGTTTAGCGCGTTTTAGAGCTAGAAA/AAAAGCACCGACTCGGTGCC	
	AF4 IVT	GAAATTAATACGACTCACTATAGGTCTCATTCCAGCAACACGTGTTTTAGAGCTAGAAA/AAAAGCACCGACTCGGTGCC	
	GATA2 IVT	CATGTAGTTGTGCGCCGTTTTAGAGCTAGA/AAAAGCACCGACTCGGTGCC	
Pluripotency Markers (qPCR)	*OCT4*	GGGTTTTTGGGATTAAGTTCTTCA/GCCCCCACCCTTTGTGTT	
	NANOG	ACAACTGGCCGAAGAATAGCA/GGTTCCCAGTCGGGTTCAC	
	SOX2	CAAAAATGGCCATGCAGGTT/AGTTGGGATCGAACAAAAGCTATT	
	REX1	CCTGCAGGCGGAAATAGAAC/GCACACATAGCCATCACATAAGG	
	CRIPTO	CGGAACTGTGAGCACGATGT/GGGCAGCCAGGTGTCATG	
	DNMT3B	GCTCACAGGGCCCGATACTT/GCAGTCCTGCAGCTCGAGTTTA	
Housekeeping gene	*GAPDH*	GCACCGTCAAGGCTGAGAAC/AGGGATCTCGCTCCTGGAA	
Off-target genomic PCR	*RNF4*	CAGACCGTGACTCCCGAAA/GTCAGCGGGGAACAAAAACC	
	RHOT2	TGTTACTGGGCGAGGGTAGG/CTACGGCCGCTACCTGAGTA	
	FAIM2	AGGCTCGTCCCATCCTTTTG/CACATCCCCATTTGCTCCCT	
	RPL8	GCAGGCAGTTCTAGAAGCCA/CCTTAGTTATCTGGATTTCCAGAAC	
	BTNL8	TAGGAGTCTTGGTGGTGTTCAT/ATATCGTGGCACCTGGCTAC	
	MYBL2	GCAGTCGGAGGAAGTGACAA/CTCCTGGCCCCTCTTAGACT	
Mycoplasma PCR	Nature	TGCACCATCTGTCACTCTGTTAACCTC/GGGAGCAAACAGGATTAGATACCCT	
	M1	ACACCATGGGAGCTGGTAAT/CTTCATCGACTTTCAGACCCAAGGCAT	

a substrate for chemiluminescence. Probes were synthesized by PCR using the PCR DIG Probe Synthesis Kit (Roche Diagnostics). 5′ probe was generated using plasmidic DNA and 3′ probe using genomic DNA as a templates. Primers used for probes are detailed in Table 2.

T7 endonuclease assay

iCas9-FL-BCP-hiPSCs were treated with doxycycline (2 μg/ml) for two days before and during transfection. Cells were dissociated with Accutase (Stem Cell Technologies) and 200.000 cells were electroporated with 120 pmol of a single IVT-gRNA (*MLL*, *GATA2* or *AF4*). Genomic DNA was extracted four days after gRNA transfection. Genomic regions flanking the CRISPR target sites were PCR amplified (Table 1). PCRs were denatured and re-annealed and then PCRs were treated with 5U of T7EI at 37 °C for 1 h.

In vitro transcription

T7 RNA polymerase promoter was added to gRNA sequences by PCR using as a template the pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene #48138) containing the guide RNA sequences for *MLL*, *GATA2* or *AF4*. PCR amplification were performed using specific forward primers and a universal reverse primer (Table 2). PCR products were used as templates for IVT using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs). The resulting gRNAs were purified using the MEGAclear kit (Life Technologies), eluted in RNase-free water and stored at −80 °C until use.

Mycoplasma test

Primers used are listed in Table 2. PCR conditions were:

94 °C: 20″
×40 cycles
  94ªC: 20″
  55 °C: 20″ (Nature primer) or 58 °C: 10″ (M1 primer)
  65ªC: 20–40″

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.04.011.

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