Stem Cell Research 23 (2017) 105–108

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Stem Cell Line

Generation of an OCT4 reporter human induced pluripotent stem cell line using CRISPR/SpCas9

Diego Balboa [a,*], Jere Weltner [a], Yuval Novik [a], Solja Eurola [a], Kirmo Wartiovaara [a,b], Timo Otonkoski [a,c]

[a] Research Programs Unit, Molecular Neurology and Biomedicum Stem Cell Centre, Faculty of Medicine, University of Helsinki, 00014 Helsinki, Finland
[b] Clinical Genetics, HUSLAB, Helsinki University Central Hospital, 00290 Helsinki, Finland
[c] Children's Hospital, Helsinki University Central Hospital, University of Helsinki, 00290 Helsinki, Finland

ARTICLE INFO

Article history:
Received 30 May 2017
Received in revised form 29 June 2017
Accepted 7 July 2017
Available online 11 July 2017

ABSTRACT

OCT4 is a crucial transcription factor in the pluripotent stem cell gene regulatory network and an essential factor for pluripotent reprogramming. We engineered the previously reported HEL24.3 hiPSC to generate an OCT4 reporter cell line by knocking-in a T2A nuclear EmGFP reporter cassette before the OCT4 gene STOP codon sequence. To enhance targeted insertion, homologous recombination was stimulated using targeted cutting at the OCT4 STOP codon with CRISPR/SpCas9. This HEL24.3-OCT4-nEmGFP cell line faithfully reports endogenous OCT4 expression, serving as a useful tool to examine temporal changes in OCT4 expression in live cells during hiPSC culture, differentiation and somatic cell reprogramming.

Resource table

Unique stem cell line identifier	UHi001-B
Alternative name(s) of stem cell line	HEL24.3-OCT4-nEmGFP; HEL24.3-OCT4-nEmGFP-A-G3
Institution	Biomedicum Stem Cell Center, University of Helsinki
Contact information of distributor	Diego Balboa
Type of cell line	hiPSC
Origin	HEL24.3 hiPSC described in Trokovic et al., 2015
Additional origin info	Sex: Male
Cell Source	Human foreskin fibroblasts (HFFs; CRL-2429, ATCC)
Method of reprogramming	CytoTuneTM-iPS Sendai Reprogramming Kit (Life Technologies)
Genetic Modification	YES
Type of Modification	Reporter knock-in
Associated disease	N/A
Gene/locus	OCT4 (POU5F1)
Method of modification	CRISPR/SpCas9
Name of transgene or resistance	T2A-NLS-EmGFP-P2A-Pac
Inducible/constitutive system	N/A
Date archived/stock date	N/A
Cell line repository/bank	N/A
Ethical approval	Coordinating Ethics Committee of Helsinki and Uusimaa Hospital District approved generation and use of human iPSC (statement nr. 423/13/03/00/08) on April 2009. Human foreskin cells obtained from ATCC (HFFs; CRL-2429)

Resource utility

We engineered an OCT4-nEmGFP (nuclear Emerald Green Fluorescent Protein) reporter hiPSC that enables the examination of OCT4 expression in live hiPSC cells during stem cell maintenance, differentiation and reprogramming to pluripotency.

Resource details

OCT4 (also known as POU5F1 or Oct-3/4) is a transcription factor crucial for the formation, self-renewal and differentiation of pluripotent stem cells in the mammalian embryo. It is also a critical component of the transcription factors set used for reprogramming of somatic cells to pluripotent stage (Takahashi and Yamanaka, 2006).

We used the previously described human induced pluripotent stem cell line (hiPSC) HEL24.3 (Trokovic et al., 2015) to generate an OCT4 reporter by knocking-in a T2A-NLS-EmGFP-P2A-Pac cassette at the endogenous OCT4 locus STOP codon sequence. Homologous recombination was stimulated using CRISPR/SpCas9 with a OCT4 STOP codon-targeted gRNA (Fig. 1A. nEmGFP reporter cassette targeting strategy: 2A self-cleaving sequences were used to generate a multicistronic reporter cassette that enables expression of nuclear localized EmGFP (NLS) and puromycin resistance gene Pac (Puro), allowing for positive selection of correctly targeted clones. Red and black arrowheads depict cutting gRNA binding site and screening PCR primers binding sites, respectively). The reporter cassette donor template was delivered by electroporation as a closed circular plasmid, together with SpCas9-expressing plasmid and a gRNA PCR transcriptional

* Corresponding author.
E-mail address: diego.balboa@helsinki.fi (D. Balboa).

http://dx.doi.org/10.1016/j.scr.2017.07.006

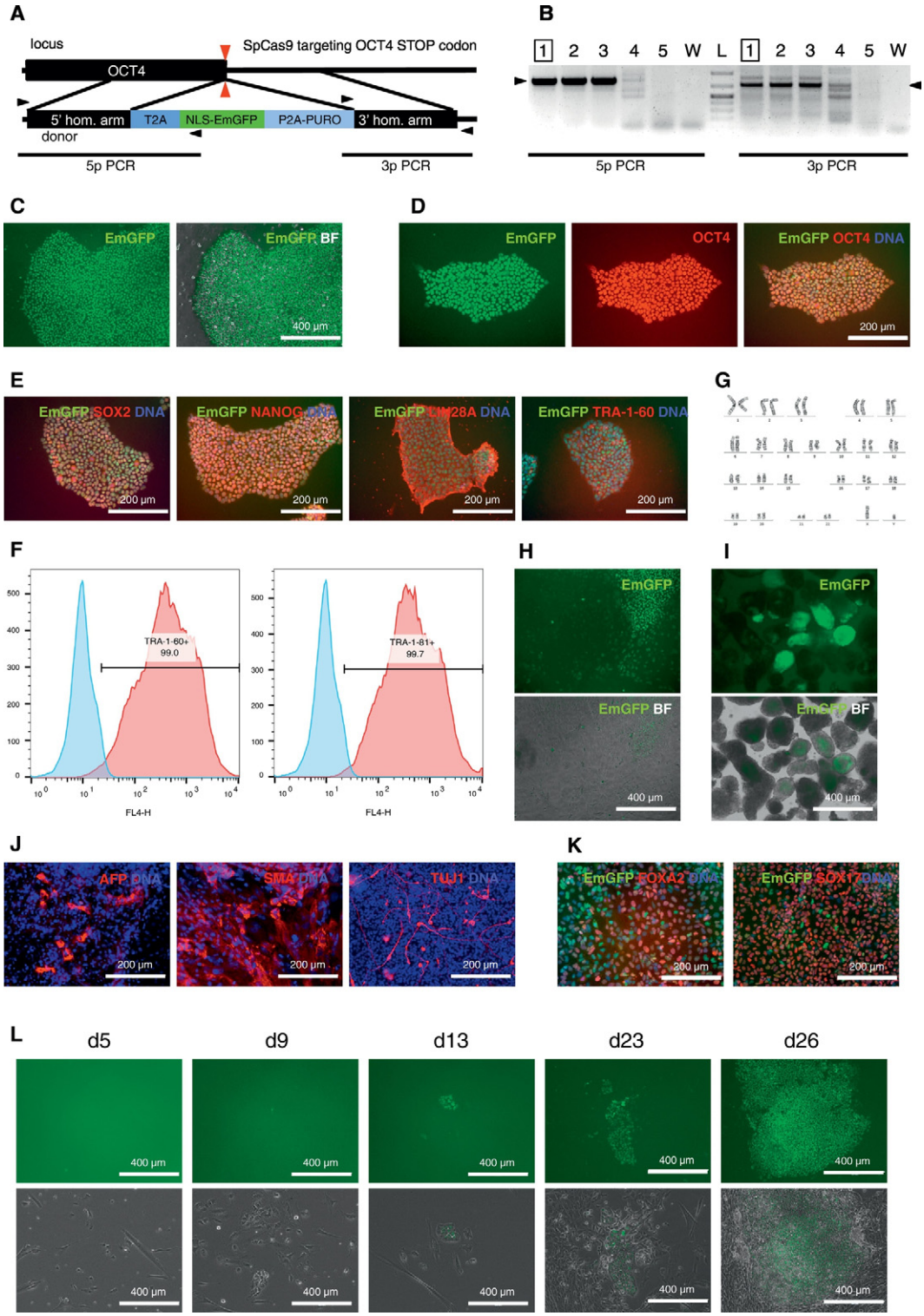

Fig. 1. Generation of an OCT4 reporter hiPSC using CRISPR/SpCas9.

cassette. Clones resistant to puromycin were individually picked, expanded and screened for correct integration by PCR (Fig. 1B. Screening of reporter cassette recombination in the OCT4 locus by PCR using primers spanning 5′ and 3′ homology arms as depicted in Fig. 1A. Primers sequences detailed in Table 2. Clones 1,2 and 3 are correctly targeted, while 4 and 5 are not targeted (W, water control; L, size ladder). Clone 1 was further single-cell sorted to ensure complete clonality of the reporter hiPSC. Heterozygous insertion of the reporter cassette was confirmed by PCR, while the non-targeted allele was intact (data not shown).

HEL24.3-OCT4-nEmGFP reporter hiPSCs express bright nuclear EmGFP in live imaging (Fig. 1C. EmGFP fluorescence and overlaid brightfield (BF) image of a reporter colony by live microscopy). Characterization by immunocytochemistry showed overlapping of EmGFP signal with OCT4 (Fig. 1D), SOX2 and NANOG staining in nuclei, with LIN28A in the cytoplasm, and with TRA-1-60 in the cell membrane

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel C
Phenotype	Immunocytochemisty	Staining for OCT4, SOX2, NANOG, TRA1-60 and LIN28.	Fig. 1 panel D and E
	Flow cytometry	TRA-1-60 and TRA-1-81	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46, XY Resolution approx. 400 bands	Fig. 1 panel G
Identity	Microsatellite PCR (mPCR)	Not performed	–
	STR analysis	StemElite ID System, 9 sites	–
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous insertion of reporter cassette in OCT4 locus determined by PCR SpCas9 putative off-target sites analyzed by Sanger sequencing	Data with author –
	Southern Blot OR WGS	–	–
Microbiology and virology	Mycoplasma	Biochemical luminescence MycoAlert™ Mycoplasma Detection Kit, Lonza. Negative	Data with author
Differentiation potential	Embryoid body formation	Spontaneous embryoid body differentiation. Directed differentiation to endoderm. Three lineage tissues in teratoma assay.	Fig. 1 panel H,I, J and K
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	–	–
Genotype additional info (OPTIONAL)	Blood group genotyping	–	–
	HLA tissue typing	–	–

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:500	Santa Cruz Biotechnology Cat# sc-9081, RRID:AB_2167703
	Rabbit anti-SOX2	1:500	Cell Signaling Technology Cat# 3579S, RRID: AB_2195767
	Rabbit anti-NANOG	1:500	Cell Signaling Technology Cat# 4903P, RRID:AB_10829232
	Rabbit anti-LIN28	1:500	Cell Signaling Technology Cat# 3695P, RRID:AB_10886920
	Mouse anti-TRA1-81	1:50, used for FACS	Thermo Fisher Scientific Cat# MA1-024, RRID:AB_2536706
	Mouse anti-TRA1-60	1:50, used also for FACS	Thermo Fisher Scientific Cat# MA1-023, RRID:AB_2536699
Germ layers	Rabbit anti-AFP	1:500	Dako Cat# A0008, RRID:AB_2650473
	Mouse anti-SMA	1:400	Sigma-Aldrich Cat# A2547, RRID:AB_476701
	Mouse anti-TUJ1	1:500	R and D Systems Cat# MAB1195, RRID:AB_357520
	Goat anti-FOXA2	1:500	Santa Cruz Biotechnology Cat# sc-9187, RRID:AB_2104886
	Goat anti-SOX17	1:500	R and D Systems Cat# AF1924, RRID:AB_355060
Secondary antibodies	Donkey anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor® 594 conjugate	1:500	Thermo Fisher Scientific Cat# A21207, RRID:AB_141637
	Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1:500	Thermo Fisher Scientific Cat# A-21203, RRID:AB_2535789
	Donkey anti-Goat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1:500	Thermo Fisher Scientific Cat# A-11058, RRID:AB_2534105
	Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	1:500, used for FACS	Thermo Fisher Scientific Cat# A-31571, RRID:AB_162542

	Target	Forward/Reverse primer (5′-3′)
Primers		
OCT4 stop codon SpCas9 gRNA oligo	OCT4 stop codon	GTGGAAAGGACGAAACACCgCCTGTCCCCCATTCCTAGAAgttttagagctaGAAAtag
OCT4 T7 test PCR	OCT4 stop codon	OCT4_nmCas9cut_F3: CCCCATTTTGGTACCCCAGG OCT4_nmCas9cut_R3: CCAGCTCTCCCTCTGATGTTG Amplicon = 520 bp
Insertion detection PCR 5′homology arm	OCT4 and EmGP	OCT4 v1/2 sg hum F: GAAAGCGAACCAGTATCGAG eGFP_screen_Rv: GAACAGCTCCTCGCCCTTG Amplicon = 841 bp
Insertion detection PCR 3′homology arm	Pac and OCT4	Puro_Fw: CACCAGGGCAAGGGTCTG OCT4_3UTR_R1: CTCTGGGGCAAAGACCCCTA Amplicon = 884 bp
Plasmids		
pUC19-OCT4-T2A-NLS-EmGFP-P2A-Puro	PCR-cloning	Deposited in Addgene
CAG-Cas9	Cloned from PX330 (Addgene Plasmid #42230)	Deposited in Addgene

(Fig. 1E). Flow cytometry confirmed the expression of pluripotency markers TRA-1-60 and TRA-1-80 (Fig. 1F). Karyotype was normal 46, XY (Fig. 1G) and STR genotyping demonstrated its derivation from HEL24.3. (See Table 1.)

We tested the temporal expression of nEmGFP upon spontaneous differentiation in monolayer and in embryoid-body assay (Fig. 1H and 1I. Live microscopy of EmGFP and brightfield (BF) in monolayer (Fig. 1H) and embryoid-bodies (Fig. 1I)), resulting in a gradual decrease of EmGFP fluorescence in the differentiated cells. OCT4-nEmGFP reporter cells were capable of differentiating to the three germ layers by embryoid body assay, demonstrating their pluripotency (Fig. 1J). AFP, endoderm marker alpha fetoprotein; SMA, mesoderm marker smooth muscle actin; TUJ1, neuroectoderm marker tubulin beta III). Directed differentiation of the reporter cells to definitive endoderm resulted in EmGFP fluorescence decrease in cells expressing endoderm markers FOXA2 and SOX17 (Fig. 1K).

Fibroblast-like cells were derived from HEL24.3-OCT4-nEmGFP by prolonged culture of plated embryoid-bodies. We reprogrammed these cells back to pluripotency using replicative episomal plasmid technology and monitored daily the appearance of epithelial colonies and expression of EmGFP during the reprogramming process. First epithelial colonies were detected around day 13, already expressing nuclear EmGFP (Fig. 1L. Live microscopy for EmGFP and brightfield (BF) at different time points of the reprogramming process). From that timepoint onwards, reprogrammed cells formed colonies with the characteristic morphology of induced pluripotent stem cells that also expressed bright nEmGFP as a result of the endogenous OCT4 locus reactivation.

Materials and methods

hiPSC maintenance and differentiation were performed as described before (Balboa et al., 2015; Saarimäki-Vire et al., 2017; Balboa et al., 2017).

Knock-in of reporter cassette in HEL24.3 OCT4 locus

Donor template pUC19-OCT4-T2A-NLS-EmGFP-P2A-Puro was cloned by PCR. EmGFP sequence was cloned from pcDNA™6.2/N-EmGFP-DEST vector (V35620, Thermo Fisher). Homology arm lengths are 276 bp for 5′-homology-arm and 444 for 3′-homology-arm.

To stimulate homologous recombination of the donor template in the OCT4 STOP codon site, we utilized *Streptococcus pyogenes* Cas9 guide RNA-targeted endonuclease. To enable high expression in hiPSC, we cloned SpCas9 from pX330 plasmid (a gift from Feng Zhang, Addgene plasmid #42230) into a vector with a strong CAG promoter (CAG-Cas9, deposited in Addgene). We used Benchling (https://benchling.com/academic) to design SpCas9 guide RNAs targeting OCT4 STOP codon sequence. Guide RNA transcriptional cassettes were assembled by PCR as described elsewhere (Balboa et al., 2015).

2 μg pUC19-OCT4-T2A-NLS-EmGFP-P2A-Puro circular closed plasmid, 6 μg CAG-Cas9 plasmid and 0.5 μg of OCT4 stop codon SpCas9 gRNA PCR product were electroporated to two million HEL24.3 cells and plated onto Matrigel-coated plates on E8 medium containing 5 μM ROCK inhibitor (Y-27632 2HCl, Selleckchem). Electroporation, expansion, screening and single-cell sorting conditions are described elsewhere (Saarimäki-Vire et al., 2017) (Balboa et al., 2017). Chromosomal G-banding karyotyping was done by Yhtyneet Medix Laboratories, Helsinki, Finland and STR analysis was performed by Genotyping Unit, Technology Centre, Institute for Molecular Medicine Finland, FIMM, Helsinki, Finland.

Immunocytochemistry and flow cytometry

Cells were fixed by incubation for 15 min with 4% PFA in PBS at room temperature. After two washes with 1× PBS, cells were permeabilized by incubation for 15 min with 0.5% Triton X-100 in 1× PBS. Cells were blocked by incubation with UltraV block (ThermoFisher) for 10 min. Staining with primary antibodies was performed in 0.1% Tween-PBS containing diluted antibodies at 4 °C overnight. Cells were incubated with secondary antibodies diluted in 0.1% Tween-PBS after 3 washes with 1× PBS. List of antibodies used is detailed in Table 2. Flow cytometry and analysis was performed as described elsewhere (Saarimäki-Vire et al., 2017).

Embryoid body generation

To generate embryoid bodies structures, reporter hiPS cells were first dissociated into single cells by incubation for 10 min with 5 μM EDTA in 1× PBS and centrifuged at 200 rcf for 2 min. Cells were reaggregated overnight by resuspending them in E8 medium containing 10 μM ROCK inhibitor, seeding them at 10^6 cells/mL in an Ultra Low Attachment plate placed in an orbital rotation platform. Next day, ROCK inhibitor-containing E8 was removed, washed once with 1× PBS and Embryoid Body (EB) medium was added (DMEM/F12 with GlutaMAX (Life Technologies # 31331-028), 20% KnockOut Serum Replacement (Life technologies #10828-028), 0.0915 mM 2-mercaptoethanol (Life technologies #31350-010), 1× Non-Essential Amino Acids (NEAA) (Life technologies #11140-035)). EB medium was replaced every two or three days. After two weeks of culture in suspension, embryoid bodies were plated in Matrigel coated plates, let to attach and cultured in the plates for additional two weeks. Plated embryoid bodies were then fixed for immunocytochemistry or passaged several times to obtain fibroblast-like cells that were used for reprogramming experiments.

Reprogramming to pluripotency

Fibroblast-like cells derived from plated embryoid bodies were passaged 8 times. To reprogram these cells back to pluripotency, delivery of episomal reprogramming plasmids method was used. Briefly, 1 million fibroblast-like cells were electroporated using Neon system (ThermoFisher) with 6 μg episomal reprogramming plasmids pCXLE-hOCT3/4-shp53, pCXLE-hSK and pCXLE-hUL (A gift from Shinya Yamanaka; Addgene plasmids #27077, #27078 and #27080). Electroporated cells were plated on Matrigel-coated plates and cultured in reprogramming media (1:1 mixture of hES and fibroblast media supplemented with 0.25 mM sodium butyrate) until colonies appeared.

References

Balboa, D., Weltner, J., Eurola, S., Trokovic, R., Wartiovaara, K., Otonkoski, T., 2015. Conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation. Stem Cell Rep. 5:448–459. http://dx.doi.org/10.1016/j.stemcr.2015.08.001.

Balboa, D., Weltner, J., Novik, Y., Eurola, S., Wartiovaara, K., Otonkoski, T., 2017. Generation of a SOX2 reporter human induced pluripotent stem cell line using CRISPR/SaCas9. Stem Cell Res. 22:16–19. http://dx.doi.org/10.1016/j.scr.2017.05.005.

Saarimäki-Vire, J., Balboa, D., Russell, M.A., Saarikettu, J., Kinnunen, M., Keskitalo, S., Malhi, A., Valensisi, C., Andrus, C., Eurola, S., Grym, H., Ustinov, J., Wartiovaara, K., Hawkins, R.D., Silvennoinen, O., Varjosalo, M., Morgan, N.G., Otonkoski, T., 2017. An activating STAT3 mutation causes neonatal diabetes through premature induction of pancreatic differentiation. Cell Rep. 19. http://dx.doi.org/10.1016/j.celrep.2017.03.055.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676. http://dx.doi.org/10.1016/j.cell.2006.07.024.

Trokovic, R., Weltner, J., Otonkoski, T., 2015. Generation of iPSC line HEL24.3 from human neonatal foreskin fibroblasts. Stem Cell Res. 15:266–268. http://dx.doi.org/10.1016/j.scr.2015.05.012.