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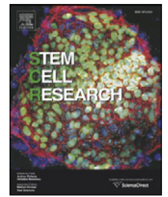

Lab Resource: Stem Cell Line

Generation of a SOX2 reporter human induced pluripotent stem cell line using CRISPR/SaCas9

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ABSTRACT

SOX2 is an important transcription factor involved in pluripotency maintenance, pluripotent reprogramming and differentiation towards neural lineages. Here we engineered the previously described HEL24.3 hiPSC to generate a SOX2 reporter by knocking-in a T2A fused nuclear tdTomato reporter cassette before the STOP codon of the SOX2 gene coding sequence. CRISPR/SaCas9-mediated stimulation of homologous recombination was utilized to facilitate faithful targeted insertion. This line accurately reports the expression of endogenous *SOX2* and therefore constitutes a useful tool to study the *SOX2* expression dynamics upon hiPSC culture, differentiation and somatic cell reprogramming.

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Resource table.

Unique stem cell line identifier	UHi001-A
Alternative name of stem cell line	HEL24.3-SOX2-ntdT; HEL24.3-SOX2-ntdT-C9-H5
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 cells (HFFs; CRL-2429, ATCC)
Method of reprogramming	CytoTuneTM-iPS Sendai Reprogramming Kit (Life Technologies)
Associated disease	Healthy donor
Gene/locus	*SOX2*
Method of modification	CRISPR/SaCas9
Gene correction	N/A
Name of transgene or resistance	T2A-NLS-tdTomato-F2A-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 generated a SOX2-ntdTomato reporter hiPSC to study the expression of SOX2 in live cells. This cell line can be utilized for monitoring SOX2 expression during stem cell maintenance, differentiation, and reprogramming of differentiated cells derived from this cell line back to pluripotency.

Resource details

SOX2 is a transcription factor that has important roles in different stages of mammalian development, especially in stem cell state acquisition and maintenance, and differentiation to neuroectodermal lineages. SOX2 is also part of the reprogramming transcription factor set, together with OCT4, KLF4 and MYC, utilized to reprogram somatic cells to the pluripotent stage (Takahashi and Yamanaka, 2006).

A SOX2-ntdTomato reporter human induced pluripotent stem cell line was generated using CRISPR/SaCas9 genome editing technology to knock-in a T2A-NLS-tdTomato-F2A-Pac reporter cassette before the STOP codon of the endogenous SOX2 locus (Fig. 1A. tdTomato reporter cassette targeting strategy. Nuclear localized tdTomato followed by F2A-puromycin enables selection of recombinant clones. Red arrowheads indicate SaCas9-cutting site. Black arrowheads indicate binding sites for PCR primers used for insertion detection). Donor template, SaCas9-expressing plasmid and gRNA-transcriptional cassette were delivered by electroporation. Puromycin-resistant clones were individually picked and screened for correct integration by PCR (Fig. 1B. PCR analysis of reporter cassette insertion in *SOX2* locus). Clone number 9

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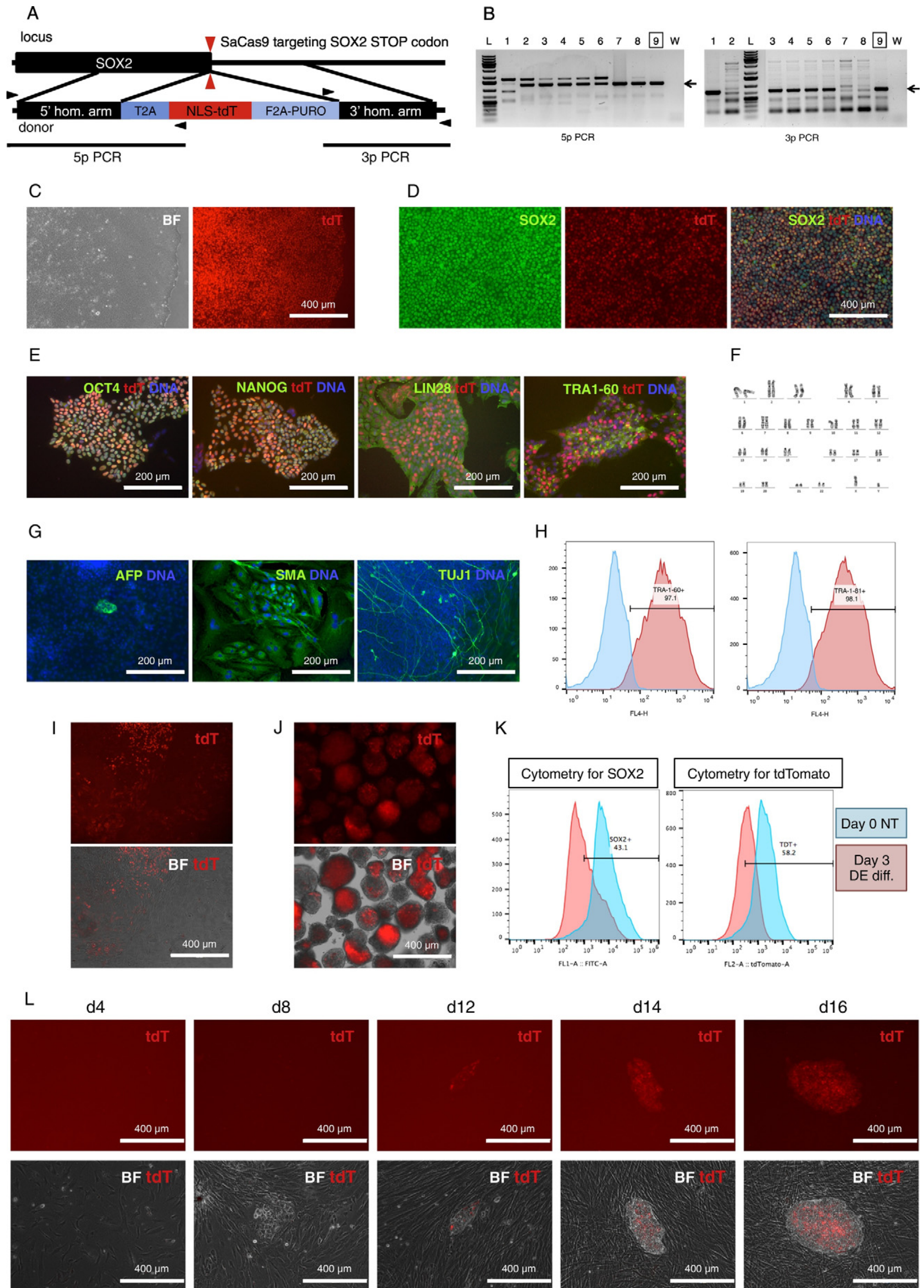

Fig. 1. Generation of a SOX2 reporter hiPSC using CRISPR/SaCas9.

Table 1
Characterization and validation of HEL24.3-SOX2-ntdT.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel C
Phenotype	Immunocytochemisty	Staining for SOX2, OCT4, NANOG, TRA1-60 and LIN28	Fig. 1 panel D and E
	Flow cytometry	TRA-1-60 and TRA-1-81	Fig. 1 panel H
		SOX2 flow cytometry	Fig. 1 panel K
Genotype	Karyotype (G-banding) and resolution	46, XY Resolution approx. 400 bands	Fig. 1 panel F
Identity	Microsatellite PCR (mPCR)	Not performed	–
	STR analysis	StemElite ID System, 9 sites	Supplementary files
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous insertion of reporter cassette in SOX2 locus determined by PCR	Data with author
		SaCas9 putative off-target sites analyzed by Sanger sequencing	Supplementary files
	Southern Blot OR WGS	–	–
Microbiology and virology	Mycoplasma	Negative	Biochemical luminescence MycoAlert™ Mycoplasma Detection Kit, Lonza. Data with author
Differentiation potential	Embryoid body formation OR Teratoma formation OR Scorecard	Spontaneous embryoid body differentiation. Directed differentiation to endoderm. Three lineage tissues in teratoma assay	Fig. 1G, I, J, K and L. See also Trokovic et al. (2015)
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	–	–
Genotype additional info (OPTIONAL)	Blood group genotyping	–	–
	HLA tissue typing	–	–

was subsequently single-cell sorted to ensure clonality of the reporter cell line. PCR-analysis confirmed reporter cassette heterozygous insertion at the SOX2 locus (data not shown).

HEL24.3-SOX2-ntdT reporter hiPSCs presented bright nuclear red fluorescent signal in live imaging (Fig. 1C, Bright field (BF) and tdTomato (tdT) live microscopy of a reporter colony). Reporter cell line characterization by immunocytochemistry for different pluripotency markers showed overlapping of the nuclear tdTomato signal with SOX2 (Fig. 1D), OCT4 and NANOG in the nuclei, with LIN28 in the cytoplasm and TRA-1-60 in the cell membrane (Fig. 1E). Flow cytometry analysis demonstrated expression of pluripotency markers TRA-1-60 and TRA-1-81 (Fig. 1H). Reporter cells were able to differentiate to three germ layers in embryoid body assay (Fig. 1G, endoderm marker alpha fetoprotein, AFP, mesoderm marker smooth muscle actin, SMA, neuroectoderm marker, tubulin beta III, TUJ1). Karyotype analysis of this clone was normal 46,XY (Fig. 1F).

We evaluated the dynamic expression of the reporter upon differentiation of HEL24.3-SOX2-ntdT cells *in vitro*. Induction of spontaneous differentiation as a monolayer or in embryoid-body assay resulted in gradual reduction in tdTomato fluorescence (BF and tdT live microscopy of differentiated cells in monolayer, Fig. 1I, or as embryoid bodies, Fig. 1J). Similarly, directed differentiation of the reporter cells to definitive endoderm showed concomitant reduction in the number of cells expressing SOX2 and tdTomato (Fig. 1K, flow cytometry of definitive

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-SOX2	1:500	Cell Signaling Technology Cat# 3579S, RRID: **AB_2195767**
	Rabbit anti-OCT4	1:500	Santa Cruz Biotechnology Cat# sc-9081, RRID: **AB_2167703**
	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	Thermo Fisher Scientific Cat# MA1–024, RRID: **AB_2536706**
	Mouse anti-TRA1-60	1:50	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**
Secondary antibodies	Donkey anti-Rabbit IgG (H + L), Alexa Fluor 488	1:500	Thermo Fisher Scientific Cat# A-21206, RRID: **AB_2535792**
	Donkey anti-Mouse IgG (H + L), Alexa Fluor 488	1:500	Thermo Fisher Scientific Cat# A-21202, RRID: **AB_2535788**

Primers		
	Target	Forward/Reverse primer (5′–3′)
SOX2 end SaCas9 gRNA oligo	SOX2 stop codon	GTGGAAAGGACGAAACACCgaaatgggaggggtgcaaaagagGTTTTAGTACTCTGGAAAC
SOX2 T7 test PCR	SOX2 stop codon	Sox2 h-m F1: GCCCTGCAGTACAACTCCAT SOX2_3′UTR_R2: tcagAAGCTTCTTCTTTTCACGTTTGCAACTG Amplicon = 964 bp
Insertion detection PCR 5′homology arm	SOX2 and tdTomato	SOX2 up_screen_Fw: CTTCGCCTGATTTTCCTCGCG tdTomato rev2: Gatgacctcctcgcccttgc Amplicon = 1263 bp
Insertion detection PCR 3′homology arm	Puro and SOX2	Puro_Fw: CACCAGGGCAAGGGTCTG SOX2_3′UTR_R1: AGACCACAGAGATGGTTCGC Amplicon = 850 bp
Plasmids		
PUC19-SOX2-T2A-NLS-tdT-F2A-PURO	PCR-cloning	Deposited in Addgene
CAG-SaCas9	Cloned from PX601 (Addgene Plasmid #61591)	Deposited in Addgene

endoderm differentiated cells for SOX2 and tdTomato, before differentiation (Day 0 NT) and after 3 days of definitive endoderm differentiation (Day 3 DE diff.))

We derived fibroblast-like cells from HEL24.3-SOX2-ntdT by plating embryoid bodies and prolonged culture. These reporter fibroblasts were then reprogrammed back to pluripotency using replicative episomal reprogramming plasmids. The reprogramming process was monitored daily for the appearance of induced pluripotent stem cell colonies. By day 8 of reprogramming the first epithelial clusters typical of reprogramming appeared and, by day 12, these clusters started to express tdTomato. This indicates that endogenous SOX2 transcriptional activation occurs already at day 12, contributing to the establishment, stabilization and expansion of the forming hiPSC colonies from that time point onwards. (Fig. 1L, tdTomato fluorescence and bright field images at different time points of the reprogramming process of HEL24.3-SOX2-ntdT-derived fibroblasts).

Materials and methods

hiPSC maintenance and differentiation

Cells were cultured with E8 medium (Life Technologies, A1517001), on Matrigel (BD Biosciences)-coated plates and passaged using 5 mM EDTA (Life Technologies, 15575-038) as a dissociation agent. Cells were spontaneously differentiated in monolayer and embryoid bodies by culturing them on hES media (KnockOut DMEM (Gibco) supplemented with 20% KO serum replacement (Gibco), 1% GlutaMAX (Gibco), 0.1 mM beta-mercaptoethanol, 1% nonessential amino acids (Gibco)) without FGF2. Embryoid bodies were generated as described elsewhere (Balboa et al., 2015). Definitive endoderm-differentiation was induced using 100 ng/mL ActivinA and 3 μM CHIR99021 (Tocris,4423) as described elsewhere (Saarimäki-Vire et al., 2017).

Gene targeting of SOX2 locus in HEL24.3

Staphylococcus aureus Cas9 was used as the guide RNA-targeted endonuclease to stimulate reporter donor template homologous recombination in the insertion site before SOX2 stop codon (Ran et al., 2015). SaCas9 was cloned from pX601 plasmid (a gift from Feng Zhang, Addgene plasmid #61591) into a vector with a strong CAG promoter to enable robust expression in hiPSC (CAG-SaCas9, deposited in Addgene). SaCas9 guide RNAs targeting SOX2 stop codon were designed using Benchling. Guide-RNA transcriptional cassettes were assembled by PCR as previously described (Balboa et al., 2015). pUC19-SOX2-T2A-NLS-tdT-F2A-PURO donor template plasmid (deposited in Addgene) was cloned by PCR. tdTomato sequence was cloned from pCSCMV:tdTomato (a gift from Gerhart Ryffel, Addgene plasmid #30530). Homology arm lengths are 951 bp for 5′-homology-arm and 478 bp for 3′-homology-arm.

Two million HEL24.3 hiPSCs were electroporated with 6 μg CAG-SaCas9 plasmid, 2 μg pUC19-SOX2-T2A-NLS-tdT-F2A-PURO donor template plasmid and 500 ng of SOX2 end SaCas9 gRNA PCR product. Electroporated cells were plated on Matrigel-coated plates on E8 containing 5 μm ROCK inhibitor (Y-27632 2HCl, Selleckchem). ROCK inhibitor was removed after 24 h, and selection started with 0.3–0.5 μg/mL puromycin 72 h after electroporation. After the cells were selected for 8 days, fluorescent colonies were picked to 96-well plates. Electroporation, expansion, screening and single-cell sorting were performed as described elsewhere (Saarimäki-Vire et al., 2017). Karyotype analysis based on chromosomal G-banding was done at Yhtyneet Medix Laboratories, Helsinki, Finland.

Immunocytochemistry and flow cytometry

Briefly, cells were fixed in 4% PFA for 15 min, 0.5% Triton X-100 in 1× PBS was used for permeabilization. Then cells were blocked with UltraV block (ThermoFisher) for 10 min, and incubated overnight at 4 °C in 0.1%-Tween-PBS-containing diluted primary antibodies. After 2–3× PBS washes, cells were incubated with secondary antibodies. See Table 2, list of antibodies. Flow cytometry was performed as described in Saarimäki-Vire et al. (2017). (See Table 1.)

Pluripotent reprogramming

Reporter iPSCs were first differentiated as EBs and plated in fibroblast medium (Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% fetal bovine serum (FBS; Life Technologies), 2 mM GlutaMAX (Life Technologies), and 100 g/mL penicillin-streptomycin (Life Technologies)). After 8 passages in culture, 1 million fibroblast-like cells were electroporated with 6 μg episomal reprogramming plasmids (pCXLE-hOCT3/4-shp53, pCXLE-hSK and pCXLE-hUL gift from Shinya Yamanaka; Addgene plasmids #27077, #27078 and #27080). Cells were plated on Matrigel-coated dishes and cultured in 1:1 mixture of hES and fibroblast media supplemented with sodium butyrate (0.25 mM; Sigma).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.05.005.

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