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Lab Resource: Genetically-Modified Single Cell Line

Generation of a human SOX10 knock-in reporter iPSC line for visualization of neural crest cell differentiation

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A B S T R A C T

SOX10 (SRY-box transcription factor 10) is not only a definitive molecular marker of neural crest cells (NCCs) but also an essential transcription factor for the differentiation of NCCs in vertebrate embryogenesis. Here, we report the establishment of a human SOX10 knock-in reporter iPSC line (SOX10-tdT) by CRISPR/Cas9-mediated homologous recombination, in which the expression of SOX10 can be monitored as tdTomato fluorescence. This iPSC line can provide a useful tool to model the differentiation process of human NCCs in vitro.

1. Resource table

Unique stem cell line identifier	KUIMSi012-A-3
Alternative name(s) of stem cell line	SOX10-tdT
Institution	Takeda-CiRA Joint program (T-CiRA), Fujisawa, Kanagawa, Japan
Contact information of the reported cell line distributor	yayoi.toyooka@cira.kyoto-u.ac.jp
Type of cell line	iPSC
Origin	Human, 1231A3 iPSC (Nakagawa et al., 2014)
Additional origin info*(applicable for human ESC or iPSC)*	Age: 29 Sex: Female Ethnicity: African/American
Cell Source	Peripheral blood
Method of reprogramming	Episomally
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	feeder-free culture
Type of Genetic Modification	Homozygous insertion of a fluorescent reporter
Associated disease	N/A
Gene/locus	SOX10, 22q13.1

(*continued on next column*)

(*continued*)

Unique stem cell line identifier	KUIMSi012-A-3
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Plasmid transfection /electroporation
All genetic material introduced into the cells	SpCas9 vector, HDR donor vector
Analysis of the nuclease-targeted allele status	Sequencing of the targeted alleles
Method of the off-target nuclease activity surveillance	N/A
Name of transgene	tdTomato, hygromycin resistance gene ($hygro^R$)
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Positive (hygromycin)
Inducible/constitutive system details	N/A
Date archived/stock date	December 2021
Cell line repository/bank	https://hpscreg.eu/cell-line/KUIMSi012-A-3
Ethical/GMO work approvals	This study was approved by the Shonan Health Innovation Park (iPark, No. CS-00001157).
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

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1.1. Manuscript section expected contents clarification Table 1

1.1.1. Resource utility

The established reporter iPSC line SOX10-tdT (KUIMSi012-A-3) visualizes SOX10 expression as tdTomato fluorescence, enabling the monitoring of in vitro differentiation of neural crest cells (NCCs) from iPSCs without fixation or antibody staining. Table 1

2. Resource details

SOX10 (SRY-box transcription factor 10) is a reliable molecular marker of NCCs and is considered a critical transcription factor for the differentiation of NCCs in vertebrate embryogenesis. To establish an iPSC line in which SOX10 expression can be visualized as tdTomato fluorescence by CRISPR/Cas9-mediated homologous recombination, we designed donor DNAs as F2A (2A self-cleaving peptides)-tdTomato-pA in combination with the PGK-hygromycin resistance gene (hygro^R)-pA cassette, which was inserted at the stop codon of the SOX10 allele (Fig. 1A). It enabled tdTomato to be transcribed and translated with SOX10 as a fusion protein under regulation of the intrinsic SOX10 promoter and then cleaved into independent proteins at the F2A site. The PGK-hygro^R-pA cassette enabled the selection of knock-in clones using hygromycin. Of the 16 clones that were picked and expanded, four were analyzed by genomic PCR for correct 5′ and 3′ junctions and integrity of the endogenous SOX10 allele to distinguish between heterozygous and homozygous transgene integration (Fig. 1B). Droplet digital PCR of the four clones was also performed to confirm heterozygous or homozygous insertion of the transgene (data not shown). Since heterozygous clones sometimes have indels in the wild-type allele, one clone (#3) in which tdTomato cDNA was homozygously inserted into SOX10 alleles was named SOX10-tdT and chosen for further characterization. Desirable insertion of tdTomato in SOX10 alleles was confirmed by sequencing the region surrounding the insertion sites (Fig. 1A). The SOX10-tdT iPSC line showed normal morphology (Fig. 1C), and karyotype analysis proved that the line had the same and normal karyotype as the parent 1231A3 iPSC line (Fig. 1D). STR analysis verified that the SOX10-tdT line had the same origin as the parent line 1231A3. The expression of pluripotent markers OCT4, SOX2, and NANOG in the undifferentiated SOX10-tdT line was confirmed by immunocytochemistry (Fig. 1E). The SOX10-tdT line was also demonstrated to be able to differentiate into cells expressing the endoderm marker SOX17, mesoderm marker Brachyury, and ectoderm marker PAX6 by inducing differentiation into the three germ layers (Fig. 1F), indicating that it maintained pluripotency after genome editing. Finally, we induced NCCs from the SOX10-tdT line (Fig. 1G) according to Kamiya et al. (2020) and confirmed that the tdTomato fluorescence was colocalized with the signal of SOX10 antibody staining in the differentiation culture (Fig. 1G), proving that the SOX10-tdT line reports intrinsic SOX10 expression correctly. Flow cytometric analysis of differentiation culture of NCCs confirmed that the cell population positive for tdTomato fluorescence was also positive for another definitive NCC marker, CD271 (p75), which indicates that the tdTomato (SOX10)-expressing cell population was NCCs (Fig. 1H). These results demonstrate that the SOX10-tdT line allows for easy monitoring of the in vitro differentiation of NCCs from iPSCs and can serve as a useful tool for studying the differentiation process of human NCCs.

3. Materials and methods

3.1. Cell culture conditions

Human iPSCs (cell line 1231A3) were cultured on dishes coated with an iMatrix-511 (Nippi) in StemFit AK03N (Ajinomoto) as described previously (Nakagawa et al. 2014).

3.2. Targeting design

The plasmid containing tdTomato cDNA was obtained from Takara Bio USA Inc. (Matz et al. 1999, Lukyanov et al. 2000). Human codon-

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel C
Pluripotency status evidence for the described cell line	Immunocytochemistry	Expression of pluripotency markers: OCT4, SOX2, NANOG (in passage 8)	Fig. 1 panel E
	Quantitative analysis	N/A	
Karyotype	Karyotype (G-banding)	46,XX,inv(9)(p12q13)	Fig. 1 panel D
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site and targeted allele-specific PCR	Homozygous insertion of tdTomato in SOX10 loci	Fig. 1 panel B
	Droplet digital PCR	Homozygous insertion of tdTomato in SOX10 loci	Not shown, but available from author
	Transgene-specific PCR	N/A	
Verification of the absence of random plasmid integration events	PCR	PCR detection specific for plasmid backbones	Supplementary figure
Parental and modified cell line genetic identity evidence	STR analysis	16 sites tested, all matched	Submitted in archive with journal
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR)	Homozygous insertion of tdTomato in Exon 4 of SOX10	Fig. 1 panel A
	PCR-based analyses	N/A	
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	N/A	
Specific pathogen-free status	Mycoplasma	MycoAlert^{TM} Mycoplasma Detection Kit, LONZA, #LT07-21, Negative (in passage 11)	Not shown, but available from author
Multilineage differentiation potential	Directed differentiation	Differentiated into derivatives of three germ layers: endoderm (SOX17 + cells), mesoderm (Brachyury + cells), ectoderm (PAX6 + cells) (in passage 12)	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	Not shown, but available from author
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

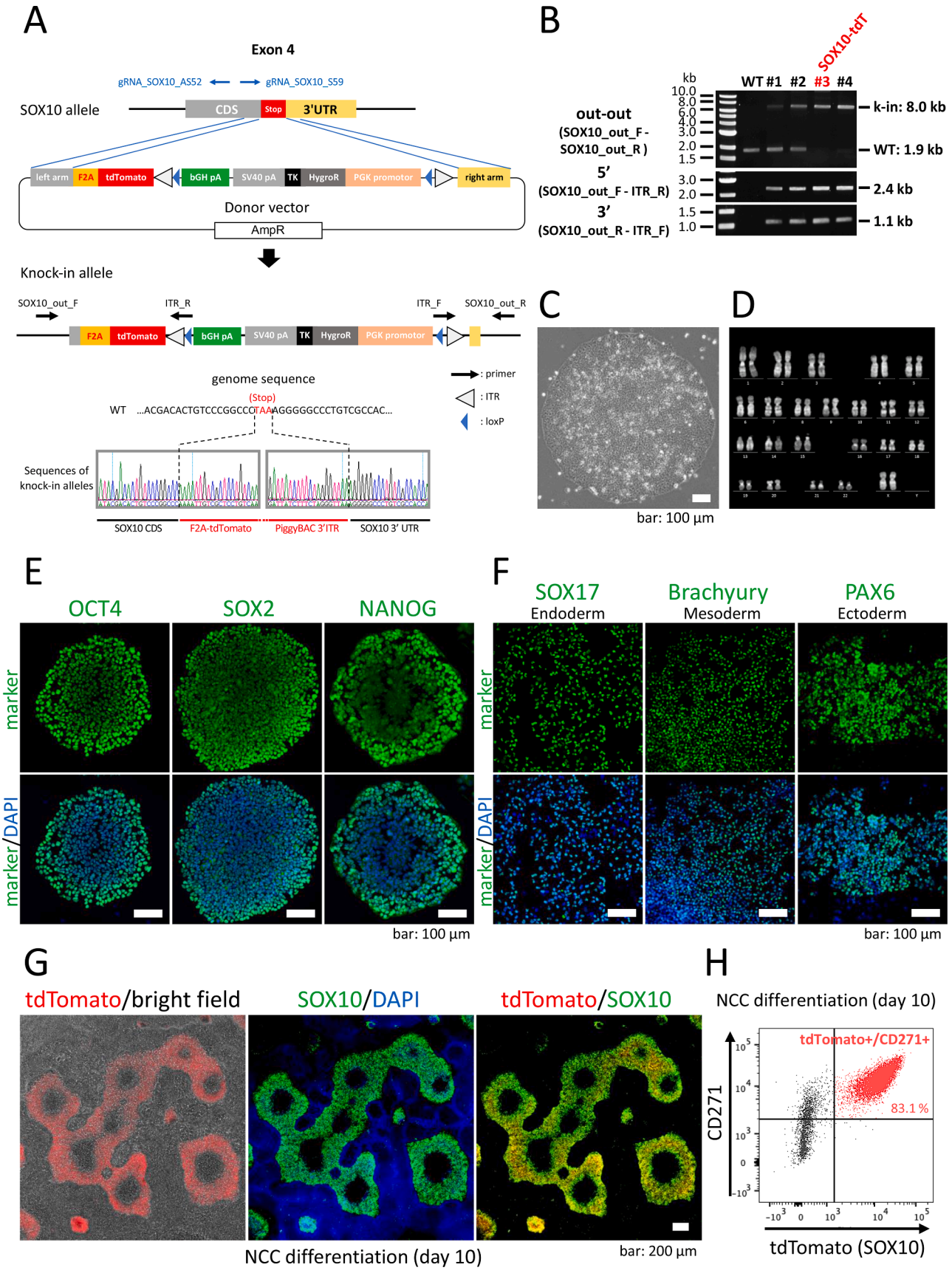

Fig. 1. Generation and characterization of SOX10-tdT iPSC line.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Rabbit anti-OCT4A	1/200	Cell Signaling Tecnology Cat # 2840,RRID: AB_2167691
	Rabbit anti-SOX2	1/200	Abcam Cat # ab92494, RRID: AB_10585428
	Rabbit anti-NANOG	1/200	Thermo Fisher Scientific Cat # PA1-097X, RRID: AB_2539868
Differentiation Markers	Goat anti-SOX17	1/100	R and D Systems Cat # AF1924, RRID: AB_355060
	Rabbit anti-Brachyury	1/600	Abcam Cat # ab209665, RRID: AB_2750925
	Rabbit anti-PAX6	1/300	Abcam Cat # ab195045, RRID: AB_2750924
Secondary antibodies	Alexa Fluor 488 Donkey anti-Rabbit IgG (H + L)	1/1000	Thermo Fisher Scientific Cat # A-21206, RRID: AB_2535792
	Alexa Fluor 488 Donkey anti-Goat IgG (H + L)	1/1000	Thermo Fisher Scientific Cat # A-11055, RRID: AB_2534102
Nuclear stain	DAPI	1 μg/mL	Thermo Fisher Scientific, Cat# D1306
NCC Marker (for FACS analysis)	Anti-Human CD271 Alexa Fluor 647-conjugated	1/5	BD Biosciences, Cat # 560326, RRID: AB_1645403
Site-specific nuclease			
Nuclease information	Nuclease type/nomenclature	SpCas9 D10A nickase vector	
Delivery method	Electroporation		
Selection/enrichment strategy	PCR/expression of hygromycin resistance gene		
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Genotyping	SOX10_out_F	GACTCATGCTGCCAAAATGTGAAA	
	SOX10_out_R	GCAGAACAGGAAAATAGGGGCAG	
	ITR_F	CACATGCGTCAATTTTACGCATGATTATC	
	ITR_R	GAGAGAGCAATATTTCAAGAATGCATGCG	
Targeted mutation analysis/sequencing	Seq 5′ recombination border (Fw1/Rv1)	CATCCACCTCACAGATCGCC/AACTCTTTGATGACCTCCTCGC	
	Seq 3′ recombination border (Fw3/Rv3)	TCCAGACTGCCTTGGGAAAAG/AGTGAGCCAGACAGAAAGCC	
Potential random integration-detecting PCRs	Ampicillin resistance gene (plasmid backbones for targeting events)	TGCAACTTTATCCGCCTCCAT/GGATGGCATGACAGTAAGAGAAT (Chen et al., 2021)	
gRNA/crRNA sequences	gRNA_ SOX10_S59	GACACTGTCCCGGCCCTAA	
	gRNA_ SOX10_AS52	GGCTGCTCCCAGTGTGTG	
Genomic target sequences	for gRNA_ SOX10_S59	GACACTGTCCCGGCCCTAAAGG (PAM)	
	for gRNA_ SOX10_AS52	CCCCACACACTGGGAGCAGCC (PAM)	

optimized spCas9 nickase harboring the D10A mutation sequence was cloned into the pCAGGS vector (Niwa et al., 1991). The homology-directed repair (HDR) donor vector, which was designed as SOX10 homology arms, was inserted into 3′ and 5′ of the F2A-tdTomato-pA in combination with the PGK-hygro^R (hygromycin resistance gene)-pA cassette of the pF2A-tdTomato-PGK-hygro^R-pA vector (Fig. 1A; synthesized at Eurofins Genomics Inc.) to facilitate the insertion of F2A-tdTomato cDNA at the stop codon of the SOX10 allele (Fig. 1A). The guide RNA (gRNA) expression vectors were constructed on a pENTR/U6 vector using the BLOCK-iT^{TM} U6 RNAi Entry Vector Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

3.3. CRISPR/Cas9-mediated knock-in

SpCas9 D10A mutant vector, two SOX10 gRNA vectors, and HDR donor vector were electroporated with 1×10^5 1231A3 iPSCs using the Neon Transfection kit (Thermo Fisher Scientific). Transfected cells were selected using 40 ng/mL hygromycin for two weeks, picked, and expanded. Desirable insertion of tdTomato in these colonies was confirmed by PCR and sequencing (Fig. 1A & B). For PCR analysis, genomic DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN) and amplified with the PrimeSTAR GXL Premix (Takara). The primers used to confirm desirable insertion are listed in Table 2.

3.4. Immunocytochemistry

The cells were fixed on the plates with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min at 4 °C, washed three times with PBS, incubated with 0.3% Triton-X100 for 30 min at room temperature (RT), and blocked with 5% bovine serum albumin/PBS for 30 min at RT. Following incubation with primary antibodies overnight at 4 °C, the cells were washed and incubated with secondary antibodies for 30 min at RT. Nuclear counterstaining was performed with DAPI. The samples were observed and photographed using BZ-X700 (Keyence). The antibodies used are listed in Table 2.

3.5. Three-germ layer differentiation

The cells were differentiated into endoderm, mesoderm, and ectoderm using the STEMdiff™ Trilineage Differentiation Kit (#05230; StemCell technologies) according to the manufacturer's instructions and were stained with germ layer-specific markers. The antibodies used are listed in Table 2.

3.6. NCC differentiation

NCC differentiation was induced according to Kamiya et al. (2020). iPSCs were seeded onto iMatrix-511-coated dishes in StemFit AK03N and cultured for 4 days, followed by culture in induction medium, StemFit AK03N without bFGF with 10 μM SB431542 and 1 μM CHIR99021 for 10 days. The cells were dissociated, stained with anti-CD271 antibody, and flow cytometric analysis was performed using an BD FACSAria Fusion Cell Sorter (BD Biosciences) and FlowJo software (BD Biosciences).

3.7. STR analysis

STR analysis was performed by checking 16 sites at the BEX Co., Ltd. (Japan).

3.8. Karyotype analysis

Karyotype analysis was performed in passage 8 by quinacrine-Hoechst staining at the chromocenter (Japan).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Yayoi Toyooka reports financial support was provided by Takeda Pharmaceutical Co Ltd.

Data availability

Data will be made available on request.

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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.2022.102696.

References

Kamiya, D., Takenaka-Ninagawa, N., Motoike, S., and Kajiya, M., Akaboshi, T., Zhao, C., Shibata, M., Senda, S., Toyooka, Y., Sakurai, H., Kurihara, H., Ikeya, M., 2020. Induction of Functional Mesenchymal Stem/Stromal Cells from Human iPCs Via a Neural Crest Cell Lineage Under Xeno-Free Conditions. Available at SSRN: https://ssrn.com/abstract=3741231 or https://doi.org/10.2139/ssrn.3741231.

Lukyanov, K.A., Fradkov, A.F., Gurskaya, N.G., Matz, M.V., Labas, Y.A., Savitsky, A.P., Markelov, M.L., Zaraisky, A.G., Zhao, X., Fang, Y.u., Tan, W., Lukyanov, S.A., 2000. Natural animal coloration can Be determined by a nonfluorescent green fluorescent protein homolog. J. Biol. Chem. 275 (34), 25879–25882.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., Lukyanov, S.A., 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat. Biotechnol. 17 (10), 969–973.

Nakagawa, M., Taniguchi, Y., Senda, S., Takizawa, N., Ichisaka, T., Asano, K., Morizane, A., Doi, D., Takahashi, J., Nishizawa, M., Yoshida, Y., Toyoda, T., Osafune, K., Sekiguchi, K., Yamanaka, S., 2014. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci. Rep. 4, 3594.

Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108 (2), 193–199.