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Lab resource: Stem Cell Line

Generation of three control iPS cell lines for sickle cell disease studies by reprogramming erythroblasts from individuals without hemoglobinopathies

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ABSTRACT

Sickle cell disease (SCD) is one of the most prevalent and severe monogenetic disorders. Previously, we generated iPS cell lines from SCD patients. Here, we generated iPS cell lines from three age-, ethnicity- and gender-matched healthy individuals as control cell lines. Cell reprogramming was performed using erythroblasts expanded from PBMC by a non-integrative method. SCD-iPSC controls expressed pluripotency markers, presented a normal karyotype, were able to differentiate into the three germ layers in embryoid body spontaneous differentiation and confirmed to be integration-free. The cell lines generated here may be used as matched healthy controls for SCD studies.

Resource table

Unique stem cell lines identifier	CBTCi002-A; CBTCi003-A; CBTCi004-A
Alternative names of stem cell lines	EB3; EB4; EB14
Institution	Hospital São Rafael – D'OR Institute for Research and Education, Salvador, Brazil
Contact information of distributor	Bruno Solano bruno.souza@hsr.com.br
Type of cell lines	iPSC
Origin	Human
Cell Source	Expanded erythroblast from human PBMC
Clonality	Clonal
Method of reprogramming	Episomal vectors carrying *OCT3/4*, shRNA p53, *SOX2*, *KLF4*, *LIN28*, *MYCL* and *EBNA1*
Multiline rationale	Age-, gender- and ethnicity-matched cell lines as unaffected controls for previously described SCD iPSCs
Gene modification	N/A
Type of modification	N/A
Associated disease	N/A
Gene/locus	N/A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	08/25/2018
Cell line repository/bank	http://biobancoipscatalogo.org/
Ethical approval	Hospital São Rafael Ethics and Research Committee CAAE 40552115000000048

Resource utility

The iPSC lines generated can be used as healthy controls in experiments conducted with the sickle-cell disease iPSC lines previously generated by our group (Martins et al., 2018). Sickle cell disease (SCD) is a monogenic disease but the mechanisms involved on its phenotypic heterogeneity remains unclear (Driss et al., 2009). It is considered a major health problem in Brazil, especially in the state of Bahia, where its prevalence is about 6% to 10% among heterozygote individuals (Cançado and Jesus, 2007), and its incidence is 1:650 in newborns (Brazilian Ministry of Health, 2014). SCD patient-derived (Martins et al., 2018) and age- and sex-matched control iPS cell lines from different ethnic origins provide useful tools for studies aiming at addressing the influence of genetic diversity in the disease pathogenesis, drug discovery and gene editing. These cell lines may also be used as normal

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
CBTCi002-A	N/A	Female	38	Afro-brazilian	N/A	N/A
CBTCi003-A	N/A	Male	19	Afro-brazilian	N/A	N/A
CBTCi004-A	N/A	Female	36	Afro-brazilian	N/A	N/A

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase-contrast images of iPSC colonies	Normal	Fig. 1, panel A
Phenotype	Qualitative analysis (immunofluorescence and PCR)	The iPSC cell lines show expression of the pluripotency markers: OCT3/4, SOX2, NANOG, TRA-1-60 (IF) and *OCT4*, *SOX2*, *KLF4*, *NANOG*, *LIN28*, *MYCL* (PCR)	Fig. 1 panel C (IF); Fig. 1 panel F (PCR)
	Quantitative analysis (flow cytometry)	CBTCCi002-A: Tra-1-60 – 94.7% CBTCCi003-A: Tra-1-60 – 98.6% CBTCCi004-A: Tra-1-60 – 96.7%	Fig. 1 panel B
Genotype	Karyotype (G-banding) and resolution	CBTCCi002-A: 46XX; CBTCCi003-A: 46XY; CBTCCi004-A: 46XX Resolution 450-500 band	Fig. 1 panel E
Identity	Microsatellite PCR (mPCR)	N/A	N/A
	STR analysis	24 loci analyzed by STR, at least 20 loci matched between parental cells and respective iPSC lines.	Data available with the authors.
Mutation analysis (if applicable)	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma by luminescence assay	Tested by Luminescence – Negative	Supplementary
Differentiation potential	Embryoid body formation	*In vitro* differentiation showing expression of *AFP*, *MSX1*, *TUBB3* (PCR) and AFP, SMA and Nestin (IF)	Fig. 1 panel I (PCR); Fig. 1 panel H (IF)
Donor screening (optional)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (optional)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

controls for studies of other hemoglobinopathies and disorders where the age, gender and ethnicity are a match.

Resource details

Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors, according to approved institutional procedures. Healthy donors were screened by hemoglobin electrophoresis, showing normal pattern. The written informed consent was obtained from all individuals. To generate the iPSCs from age and sex-matched control individuals, we delivered episomal expression cassettes of human *OCT3/4*, *SOX2*, *KLF4*, *MYCL*, *LIN28*, and shRNA of *TP53* (Okita et al., 2011) into PBMC-derived erythroblasts by nucleofection. The clones were picked, expanded and analyzed for confirmation of the pluripotency state. The iPSCs generated grew as round colonies with a typical human embryonic stem cell (hESC)-like morphology (Fig. 1A, scale bar 100 μm). The percentage of TRA-1-60$^+$ cells – a pluripotency marker - was determined by flow cytometry analysis (Fig. 1B) and confirmed by immunostaining for TRA-1-60 and other pluripotency markers - OCT3/4, NANOG and SOX2 (Fig. 1C, scale bar 25 μm). The loss of the episomal vectors used for cell reprogramming was confirmed by PCR for *EBNA1* at P20 (Fig. 1D). Chromosomal stability was confirmed by G-band karyotyping analysis at passage 15 (Fig. 1E) showing no numerical or structural clonal alterations. The expression of endogenous pluripotency genes was detected by RT-PCR (Fig. 1F). All of the iPSC lines demonstrated the ability to generate derivatives of the three-germ layers in an embryoid body (EB)-based assay. Generated EBs presented the typical morphology (Fig. 1G, scale bar 100 μm) and were positive for differentiation markers of ectoderm (Nestin), mesoderm (smooth muscle actin, alpha-SMA) and endoderm (alpha fetoprotein, AFP) (Fig. 1H, scale bar 25 μm) while undifferentiated iPSCs were negative for the differentiation markers (Supplementary Fig. 1). Evaluation of gene expression markers of endoderm (*AFP*), mesoderm (*MSX1*) and ectoderm (*TUBB3*) were also detected by RT-PCR (Fig. 1I). All cell lines were tested for *Mycoplasma* sp., showing negative results (Supplementary Fig. 2). Finally, genetic fingerprinting was performed by STR analysis, confirming the genetic identity to parental PBMCs (available with the authors) (See Tables 1 and 2.)

Materials and methods

Reprogramming of erythroblasts expanded from peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected and diluted 1:1 in 0.9% NaCl. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque®-1077 (Sigma-Aldrich). PBMCs were cultured in StemSpan supplemented with Erythroid Expansion Medium (SSEM, Stemcell Technologies), according to the manufacturer's instructions. Erythroblast expansion was confirmed by flow cytometry with anti-CD36 and anti-CD71 antibodies. Cell reprogramming was performed by nucleofection of the expanded erythroblasts with episomal plasmids encoding for *OCT4*, *SOX2*, *KLF4*, *MYCL*, *LIN28*, a short hairpin RNA for *TP53* (shP53), and *EBNA1* (Addgene plasmids #27077, #27078 and #27080, and #41857), using the Nuclelofection kit P3 solution and Nucleofector 4D, program EO-100 (Lonza). The nucleofection was performed using 2×10^6 cells and 2 μg of each plasmid. The cells were then plated in SSEM and ReproTeSR (Stem Cell Technologies) at D0. From reprogramming to D15 to D20, small colonies with an ESC-like appearance were observed. Colonies were manually picked based on the morphology and cultured in plates coated with Matrigel hESC-qualified matrix (Corning) in mTESR1 medium (Stem Cell Technologies) or Essential 8 (E8, Gibco). All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, with daily media exchanges until 80–90% confluency was achieved. The selected SCD-iPSC lines were routinely passaged using 0.5 mM EDTA or 15 mM Sodium citatre/135 mM KCl solution, once a week, with 1:10 split ratio, or twice a week with 1:6 split ratio, being cryopreserved in liquid nitrogen in mTeSR1 containing 10% DMSO.

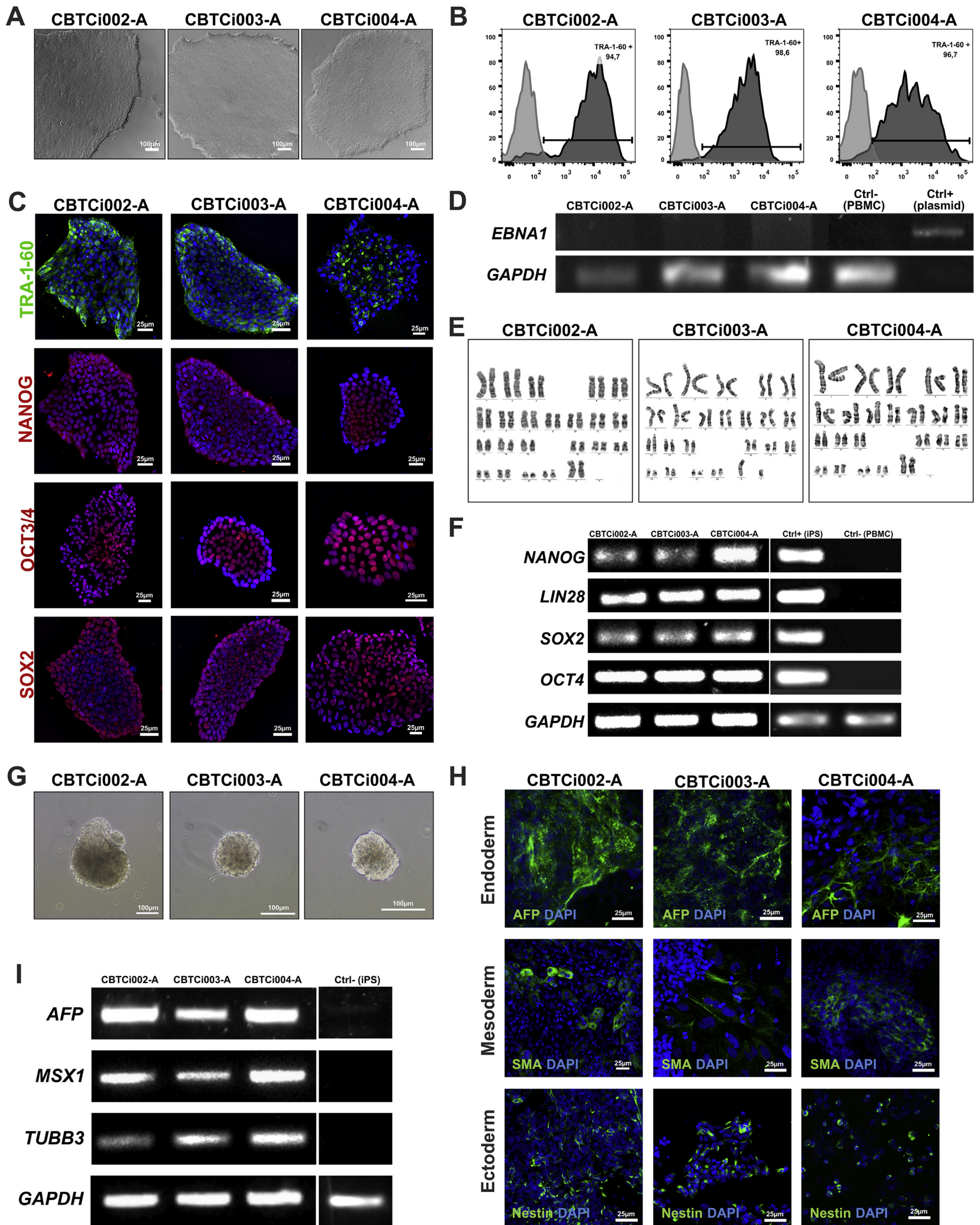

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Fig. 1. Generation and characterization of iPSCs cell lines CBTCi002-A, CBTCi003-A and CBTCi004-A obtained by reprogramming of erythroblasts expanded from three different healthy donors. (A) Phase contrast image of iPSCs. (B) Flow cytometry analysis of TRA-1-60 expression in iPSCs, by staining with anti-TRA-1-60 (dark-grey histograms) or isotype control mAb (light-grey histograms). (C) Immunofluorescence analysis of pluripotency markers. (D) Confirmation of episomal vector removal by EBNA1 PCR. (E) G-Band Karyotype of iPSCs. (F) Expression of pluripotency markers in iPSCs by RT-PCR. (G) Phase-contrast microscopy images of embryoid bodies. Expression of tri-germ layer markers on embryoid bodies evaluated by immunofluorescence (H) and RT-PCR (I).

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	TRA-1-60-Alexa Fluor 647 (mouse)	1:500	BD Biosciences Cat# 560122, RRID: AB_1645448
	Mouse IgM – Alexa Fluor 647 Isotype Control (mouse)		
	TRA-1-60 (mouse)	1:500	BD Biosciences Cat# 560806, RRID: AB_2034030
	Oct4 (rabbit)		
	Sox2 (goat)		
	Nanog (rabbit)	1:500	Abcam Cat# ab16288, RRID:AB_778563
		1:500	Abcam Cat# ab181557, RRID:AB_2687916
		1:100	Santa Cruz Biotechnology Cat# sc-17320, RRID:AB_2286684
		1:200	Abcam Cat# ab80892, RRID:AB_2150114
Differentiation Markers	AFP (rabbit)	1:300	Innovative Research Cat# 18-0055, RID:AB_138884
	SMA (mouse)	1:300	Dako Cat# M0851, RRID:AB_2223500
	Nestin (mouse)	1:300	Millipore Cat# MAB5326, RRID:AB_2251134
Secondary antibodies	Goat anti-Mouse IgM Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-21042, RRID:AB_2535711
	Goat anti-Mouse IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11017, RRID:AB_2534084
	Goat anti-Rabbit IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165
	Donkey anti-Rabbit IgG Alexa Fluor 568	1:1000	Thermo Fisher Scientific Cat# A10042, RRID:AB_2534017
	Rabbit anti-Goat IgG Alexa Fluor 568	1:1000	Thermo Fisher Scientific Cat# A-11079, RRID:AB_2534123

Primers

	Target	Forward/Reverse primer (5′–3′)
Episomal Plasmid (PCR)	*EBNA1* (244 bp)	TTT AAT ACG ATT GAG GGC GTC T GGT TTT GAA GGA TGC GAT TAA G
Pluripotency Markers (RT-PCR)	*NANOG* (78 bp)	CCT GTG ATT TGT GGG CCT G GAC AGT CTC CGT GTG AGG CAT
	LIN28 (129 bp)	AGC CAT ATG GTA GCC TCA TGT CCG C TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
	SOX2 (80 bp)	TTC ACA TGT CCC AGC ACT ACC AGA TCA CAT GTG TGA GAG GGG CAG TGT GC
	OCT4 (143 bp)	CCC CAG GGC CCC ATT TTG GTA CC ACC TCA GTT TGA ATG CAT GGG AGA GC
House-Keeping Genes (RT-PCR)	*GAPDH* (452 bp)	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA
Differentiation Markers (RT-PCR)	*TUBB3* (148 bp)	GCT CAG GGG CCT TTG GAC ATC TCT T TTT TCA CAC TCC TTC CGC ACC ACA TC
	MSX1 (307 bp)	CGA GAG GAC CCC GTG GAT GCA GAG GGC GGC CAT CTT CAG CTT CTC CAG
	AFP (281 bp)	GAA TGC TGC AAA CTG ACC ACG CTG GAA C TGG CAT TCA AGA GGG TTT TCA GTC TGG A
Targeted mutation analysis/sequencing	N/A	N/A

In vitro differentiation by embryoid body (EB) formation assay

SCD-Control-iPS cells cultured in 6 well-plates were harvested using Accutase (Gibco) for 5 min at 37 °C. Cells were washed and resuspended in 1 mL of EB medium: DMEM Knockout supplemented with 20% KOSR, 1% Non-Essential Amino Acids, 1× Glutamax, 1% Penicillin/Streptomycin (all from Thermo Fisher Scientific) and 10 μM Y-27632 (Stemcell Technologies). Next, 150 μL of a 6×10^4 cells/mL cell suspension were transferred to each well of a 96 U bottom non-adherent plate (Corning) for EB formation. Half of the media was replaced twice a week, for 2 weeks. Then, EBs were plated in 24 well-plates previously coated with Matrigel (Corning). EBs were cultured with EB medium for one week and then were fixed with PFA 4%, for immunofluorescence analysis, or incubated with Trizol (ThermoFisher Scientific), for RNA extraction and RT-PCR assays.

Immunofluorescence (IF) analysis

SCD-Control-iPS cells grown on cover slips were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 15 min at RT. Non-specific binding was blocked with Casblock (Thermofisher Scientific) in PBS for 30 min. Primary antibodies for the pluripotency markers Oct-4, Nanog, Sox2, TRA-1-60, diluted in 0.1% BSA in PBS, were added and cells were then incubated overnight at 4 °C. On the following day, the cells were incubated with secondary antibodies for 1 h at RT. Nuclei were stained with DAPI (Vector Labs). Staining for three germ layer markers on EB spontaneous differentiation assay was performed as described above, with anti-AFP, anti-SMA, and anti-Nestin antibodies. All dilutions and antibodies manufacturers are described in Table 3. Slides were observed and images were acquired using a confocal microscope, Nikon A1 (Tokyo, Japan).

Flow cytometry

SCD-Control-iPSCs were dissociated using TrypLE™ Express (Thermo Fischer Scientific), and incubated with the antibody TRA-1-60-Alexa Fluor 647 or Mouse IgM-Alexa Fluor 647 Isotype Control (BD Biosciences) diluted in PBS for 15 min at RT. The cells were washed and resuspended in PBS 1× + Hoecsht 33342 (2 µg/mL) for live cell data acquisition using a LSR Fortessa SORP flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v.X (Tristar).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from samples using TRIZOL (ThermoFisher Scientific) and 1 µg of RNA was used for cDNA synthesis using the VILO™ SuperScript™ kit according to the manufacturer's instructions. The cDNA was treated with DNAse (Ambion) and then used to perform a PCR for *LIN28*, *SOX2*, *NANOG*, OCT3/4, *AFP*, *MSX1*, *TUBB3* and the housekeeping gene *GAPDH*, using primers described at Table 3. PCR assays were performed using Platinum Taq DNA Polymerase (Invitrogen) and PCR Proflex System equipament (Applied Biosystem). Cycle parameters for *LIN28*: 94 °C 2 min, 94 °C 30 s, 65 °C *sec*, 72 °C 1 min (30×), 72 °C 10 min; *SOX2*: 94 °C 2 min, 94 °C 30 s, 62 °C sec, 72 °C 1 min (30×), 72 °C 10 min; *NANOG*: 94 °C 2 min, 94 °C 30 s, 52 °C sec, 72 °C 1 min (30×), 72 °C 10 min; *OCT3/4*: 94 °C 2 min, 94 °C 30 s, 58 °C sec, 72 °C 1 min (30×), 72 °C 10 min. Cycle parameters for *AFP*, *MSX1*, *TUBB3*: 94 °C 2 min, 94 °C 30 s, 62 °C sec, 72 °C 1 min (35×), 72 °C 10 min. PCR products were confirmed by electrophoresis on agarose gel 2%.

Short tandem repeat (STR) analysis

STR analyses were performed by pairing the parental cell source (PBMCs) of each donor and respective iPSC line. Briefly, genomic DNAs were isolated using Puregene® Core Kit A, amplified by PowerPlex Fusion System (Promega) and then analyzed with ABI3500 (Applied Biosystem) genetic analyzer using the software program GeneMapper v.5.0 (Applied Biosystem).

Karyotype analysis

Chromosomes were prepared at P15 after colcemid (10 µg/ml) overnight at 37 °C (5% CO2, 95% rH) were incubated in hypotonic solution (KCl 0.75%, 15 min, 90 °C), washed 3 min with acetic acid 5% and fixed with methanol/acetic acid (3:1). G-banded metaphase images were acquired with a 100× objective (Olympus BX61 microscope and ProgRes MFcool camera). Analysis were conducted in 20 metaphases using Lucia Karyo software (Lucia Cytogenetics) with a 400–450 band resolution.

Analysis of plasmid integration

Genomic DNA was isolated from parental PBMCs and CBTCi002-A, CBTCi003-A, CBTCi004-A using Puregene® Core Kit A (Qiagen). PCR was performed using primers specific to *EBNA1* (Table 3). The DNA was separated by 2% agarose gel electrophoresis. The DNA from parental PBMC and mixture of episomal were used as negative and positive controls respectively. *GAPDH* was used as an endogenous control. PCR assays were performed as described above. PCR conditions: 94 °C 2 min, 94 °C 30 s, 66 °C 30 s, 72 °C 1 min (35×), 72 °C 10 min.

Mycoplasma testing

Testing for mycoplasma contamination was performed using by MycoAlert detection kit (LONZA) according to the manufacturer's protocol.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101454.

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References

Brazilian Ministry of Health, 2014. Brazilian Ministry of Health. Sickle Cell Disease: What you Should Know about Genetic Inheritance [Portuguese]. Ministério da Saúde. http://bvsms.saude.gov.br/bvs/publicacoes/doenca_falciforme_deve_saber_sobre_heranca.pdf.

Cançado, R.D., Jesus, J.A., 2007. Sickle cell disease in Brazil. Rev. Bras. Hematol. Hemoter. 29 (3), 2007.

Driss, A., Asare, K.O., Hibbert, J.M., Gee, B.E., Adamkiewicz, T.V., Stiles, J.K., et al., 2009. Sickle cell disease in the post genomic era: a monogenic disease with a polygenic phenotype. Genom. Insights 2009 (2), 23–48.

Martins, G.L.S., Paredes, B.D., Azevedo, C.M., Sampaio, G.L.A., Nonaka, C.K.V., Cavalcante, B.R.R., Da Silva, K.N., Pereira, C.S.E., Soares, M.B.P., Dos Santos, R.R., Souza, B.S.F., 2018. Generation of integration-free iPS cell lines from three sickle cell disease patients from the state of Bahia, Brazil. Stem Cell Res. 33, 10–14 2018 Dec.

Okita, Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H., Yamanaka, S., et al., 2011. A more efficient method to generate integration-free human iPS cells. Nat. Methods 8 (5), 409–412.