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Lab Resource: Multiple Cell Lines

Generation of integration-free iPS cell lines from three sickle cell disease patients from the state of Bahia, Brazil

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A B S T R A C T

Sickle cell disease (SCD) is one of the most prevalent and severe monogenetic disorders, affecting several million people around the world. Clinical manifestations and complications of the disease include sickle cell pain crisis, silent cerebral infarct, stroke, nephropathy and early death. In this study, we generated induced pluripotent stem cell (iPSC) lines from three homozygous SCD patients from the state of Bahia, Brazil, where SCD is highly prevalent. Peripheral blood mononuclear cells were collected and erythroblasts were expanded for cell reprogramming with the use of non-integrative episomal vectors. The generated iPSC lines expressed high levels of pluripotency markers, presented a normal karyotype and were able to differentiate into the three germ layers in embryoid body spontaneous differentiation assays. Moreover, the expression of the episomal vectors was lost in all iPSC lines after 15 passages. These iPSC lines may help increasing the knowledge about SCD pathogenesis and can be a useful tool for drug testing and gene editing studies.

Resource table

Unique stem cell lines identifier	CBTCi005-A; CBTCi006-A; CBTCi007-A
Alternative names of stem cell lines	EB5; EB8; EB13
Institution	Hospital São Rafael – Centro de Biotecnologia e Terapia Celular
Contact information of distributor	Bruno Solano bruno.souza@hsr.com.br
Type of cell lines	hiPSC
Origin	Human
Cell Source	Expanded erythroblast from human PBMC
Clonality	Clonal
Method of reprogramming	Episomal vectors with the oriP/EBNA-1 backbone from Addgene OCT3/4-shp53F #27077; SOX2 and KLF4 #27078; LIN28 and L-MYC #27080; EBNA1 #41857
Multiline rationale	Same disease non-isogenic cell lines
Gene modification	N/A
Type of modification	N/A
Associated disease	Sickle Cell disease
Gene/locus	HBB 11p15.4 Chr11: 5227002 (on Assembly GRCh38) Chr11: 5248232 (on Assembly GRCh37) NM_000518.4(HBB):c.20A > T (p.Glu7Val)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	N/A
Cell line repository/ bank	N/A
Ethical approval	Hospital São Rafael Ethics and Research Committee CAAE 40552115000000048

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1. Resource utility

Sickle cell disease (SCD) is a monogenic disease but the mechanisms involved in its phenotypic heterogeneity remains unclear (Driss, 2009). It's considered a major problem in Brazil, especially in the state of Bahia, where its prevalence is among 6% to 10% of heterozygote individuals (Cançado and Jesus, 2007) and its incidence is 1 among 650 in newborn (Brazilian Ministry of Health, 2014). SCD patient-derived iPSCs 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.

2. Resource details

Peripheral blood mononuclear cells (PBMCs) were donated from patients with homozygous sickle cell disease, according to approved institutional procedures. The written informed consent was obtained from all individuals. To generate the iPSCs from SCD patients, we delivered episomal expression cassettes of human OCT3/4, SOX2, KLF4, L-MYC, LIN28, and shRNA of TP53 (Okita Okita et al., 2011) into PBMC-derived erythroblasts by nucleofection. Individual clones were picked, expanded and analyzed at the cellular and genetic levels to confirm successful reprogramming. After 30 days, the colonies displayed a typical human embryonic stem cell (hESC)-like morphology with refractive edges, as seen by bright field (BF) microscopy (Fig. 1A, scale bar 100 μm). Pluripotency was assessed by flow cytometry for pluripotent stem cell marker TRA-1-60 (Fig. 1B) and by immunofluorescence assay to TRA-1-60, Oct3/4, Nanog and Sox2 (Fig. 1C, scale bar 25 μm). The expression of endogenous pluripotency genes was detected by RT-PCR (Fig. 1D). Sickle cell point mutation in HBB gene was present in all three selected clones (Fig. 1E). After 15 passages, all of the iPSCs were negative for EBNA-1 expression, as evaluated by PCR, demonstrating the loss of episomal vectors (Fig. 1F). The iPSC lines displayed normal karyotypes at passage 15 (Fig. 1G). To test the ability of the hiPSC lines to generate derivatives of three germ layers *in vitro*, the embryoid body (EB)-based assay was performed. EBs from each clone presented regular morphology (Fig. 1H, scale bar 100 μm). The expression of specific markers of endoderm (AFP), mesoderm (MSX1) and ectoderm (TUBB3) markers was detected by RT-PCR (Fig. 1J) Spontaneously differentiated cells were immunostained for differentiation markers, such as Nestin for ectoderm, smooth muscle actin (SMA) for mesoderm and alpha fetoprotein (AFP) for endoderm (Fig. 1I, scale bar 25 μm). The absence of contamination with *Mycoplasma* sp. was also demonstrated (Supplementary Fig. 1). Finally, genetic fingerprinting through STR analysis was performed, confirming genetic identity to parental PBMCs (available with the authors).

3. Materials and methods

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

Peripheral blood was collected and diluted 1:1 in saline 0.9%. PBMCs separated by density gradient using Ficoll-Paque®-1077 (Sigma-Aldrich) centrifugation. Freshly isolated PBMCs were seeded in StemSpan supplemented with Erythroid Expansion Medium (SSEM, Stemcell Technologies) according to the manufacturer's instructions. Non-adherent cells were collected and the adequate expansion of erythroblasts was confirmed by flow cytometry using CD36 and CD71 positive markers. Expanded erythroblasts were collected and nucleofected with a mixture of episomal plasmids encoding hOCT4, hSOX2, hKLF4, hL-MYC, hLIN-28, a short hairpin RNA for TP53 (shP53) and EBNA-1 (Addgene plasmids #27,077, #27,078 and #27,080, and #41,857) using Nuclelofection kit P3 solution and Nucleofector 4D, program EO-100 (Lonza). Transfected cells were plated in SSEM and ReproTeSR (Stemcell Technologies) media as described in the manufacturer's manual. At D15 to D20, small colonies with an ES-like appearance were observed. Colonies were manually picked based on the morphology between D20 to D30 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 cultured at 37 °C in humidified atmosphere containing 5% CO_2, feeding daily with pluripotent stem cell medium until the cells reached 80–90% confluency. The selected SCD-iPSC lines were routinely passaged using 0.5 mM EDTA or 15 mM Sodium citatre / 135 mM KCl solution and frozen in liquid nitrogen in mTeSR1 containing 10% DMSO and thawed in the presence of 10 μM Y-27632 (Stemcell Technologies) for 24 h for further characterizations (Tables 1 and 2).

3.2. In vitro differentiation by embryoid body (EB) formation assay

SCD-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) plus 10 μM Y-27632 (Stemcell Technologies). Then, the volume was adjusted to achieve a suspension of 6×10^4 cells/mL. From this cell suspension, 150 μL were plated in 96 U bottom non-adherent wells (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 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.

3.3. Immunofluorescence (IF) analysis

SCD-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 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 were added and cells were then incubated overnight at 4 °C. After washing with PBS for three times, 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, using antibodies against AFP for endoderm, SMA for mesoderm and Nestin for ectoderm. All dilutions and antibodies manufacturers are described in Table 3. Slides were analyzed using a confocal microscope, Nikon A1 (Tokyo, Japan).

3.4. Flow cytometry

SCD-iPSCs were dissociated using TrypLE™ Express (Thermo Fischer Scientific), collected and prepared for incubation with the antibody TRA-1-60-Alexa Fluor 633 (BD Biosciences) for 15 min at RT. The cells were washed and resuspended in PBS 1× + Hoecsht 33,342 (2 μg/mL) for live cell data acquisition using a LSR Fortessa SORP flow cytometer

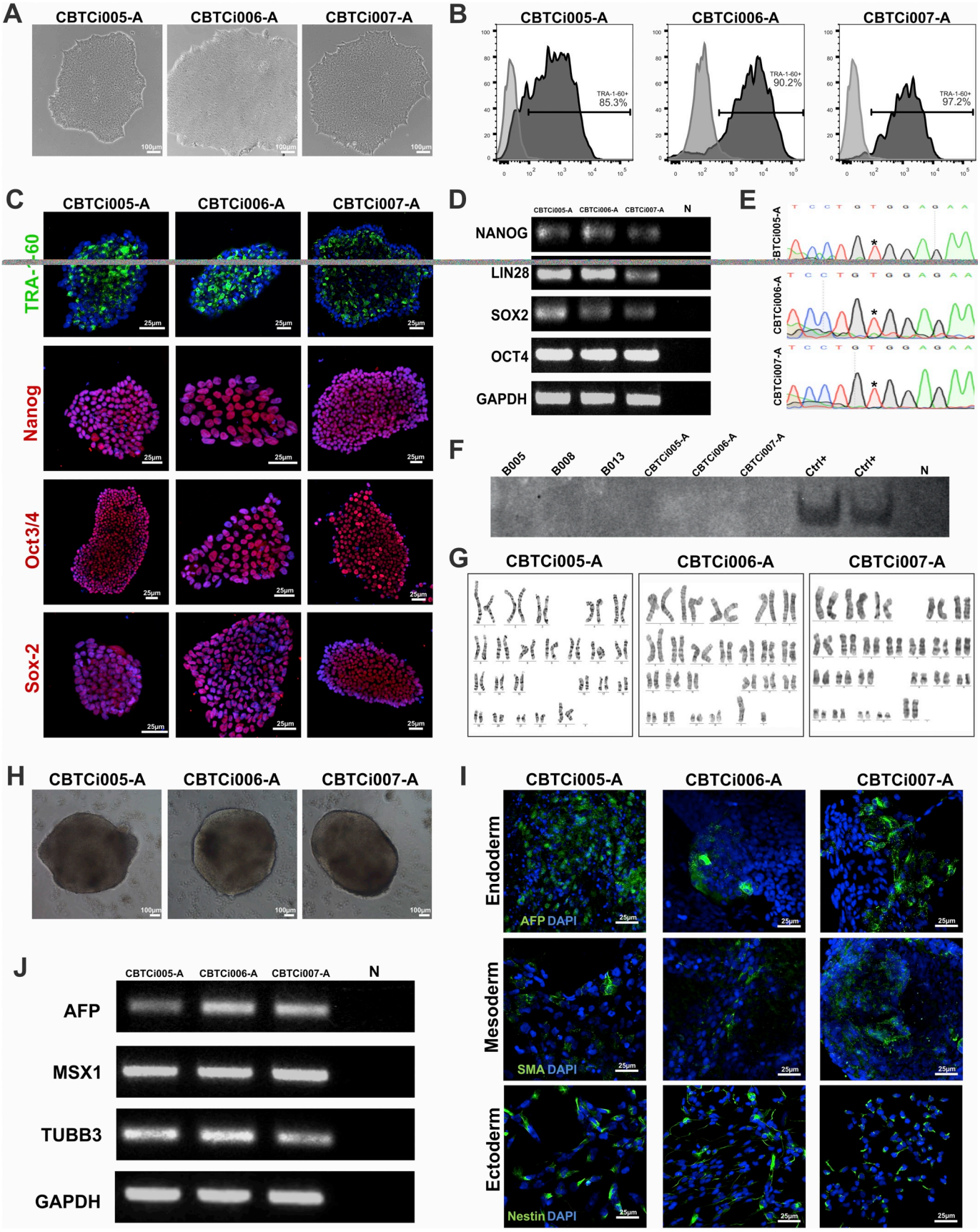

Fig. 1. Generation and characterization of iPSCs cell lines CBTCi005-A, CBTCi006-A and CBTCi007-A obtained by expanded erythroblast reprogramming from 3 different sickle cell disease patients.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
CBTCi005-A	N/A	Female	38	Afro-brazilian	HBB (11p15.4)	Sickle cell
CBTCi006-A	N/A	Male	19	Afro-brazilian	HBB (11p15.4)	Sickle cell
CBTCi007-A	N/A	Female	36	Afro-brazilian	HBB (11p15.4)	Sickle cell

(BD Biosciences). Data analysis was performed using FlowJo v.X (Tristar).

3.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from samples using TRIZOL reagent (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 performed a PCR for LIN28, SOX2, NANOG, POU5F1, AFP, MSX1 TUBB3 and the housekeeping gene GAPDH, using primers described at Table 3. PCR products were confirmed by electrophoresis on agarose gel 2%.

3.6. Sequencing analysis

Genomic DNA was extracted and amplified by PCR using a set of primers targeting exons 1 and 2 of HBB gene. PCR products were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent kit (Applied Biosystems, Foster City, CA, USA) following the manufacture's guidelines. The amplicons were then sequenced in both directions using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and the manufacturer's instructions. Thereafter, the cycle sequencing product was purified using the BigDye® XTerminator™ purification kit (Applied Biosystems) and the manufacturer's protocol was followed for the purification steps. Finally, the purified cycle sequencing PCR product was subjected to capillary sequencing in an ABI 3500 Genetic Analyzer (Applied Biosystems).

3.7. Short tandem repeat (STR) analysis

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

3.8. 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 metaphases were photographed at 100× (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.

3.9. Analysis of plasmid integration

Genomic DNA was isolated from parental PBMCs and CBTCi005-A, CBTCi006-A, CBTCi007-A using Puregene® Core Kit A (Qiagen). PCR was performed using primers specific to EBNA-1 (Table 3). The DNA was separated by 8% polyacrylamide gel electrophoresis. The DNA from parental PBMC and a mixture of episomal plasmids were used as negative and positive controls respectively.

3.10. Mycoplasma testing

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

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase-contrast images of iPSC colonies and embryoid bodies	Normal	Fig. 1 panel A (iPSC colonies); Fig. 1 panel H (embryoid bodies)
Phenotype	Qualitative analysis (immunofluorescence and PCR)	The iPSC cell lines show expression of the pluripotency markers: Oct4, Sox2, Nanog, Tra-1-60 (IF) and Oct4, Sox2, KLF4,Nanog, Lin28, L-Myc (PCR)	Fig. 1 panel C (IF); Fig. 1 panel D (PCR)
	Quantitative analysis (flow cytometry)	EB5: Tra-1-60 - 85,3% EB8: Tra-1-60 - 92,0% EB13: Tra-1-60 - 97,2%	Fig. 1 panel B
Genotype	Karyotype (G-banding) and resolution	CBTCCi005-A: 46XX; CBTCCi006-A: 46XY; CBTCCi007-A: 46XX Resolution 450–500 band	Fig. 1 panel G
Identity	Microsatellite PCR (mPCR)	N/A	N/A
	STR analysis	24 loci analyzed by STR, all matching. 22 of them are listed.	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Homozygous c.20A > T (p.Glu7Val) in PBMC and iPSC	Fig. 1 panel E
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma by luminescence assay	Tested by Luminescence – Negative	Supplement document
Differentiation potential	Embryoid body formation	*In vitro* differentiation showing expression of AFP, MSX, TUBB3 (PCR) and AFP, SMA and Nestin (IF)	Fig. 1 panel J (PCR); Fig. 1 panel I (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

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	TRA-1-60 (mouse)	1:500	Abcam Cat# ab16288, RRID:AB_778563
	Oct4 (rabbit)	1:500	Abcam Cat# ab181557, RRID:AB_2687916
	Sox2 (goat)	1:100	Santa Cruz Biotechnology Cat# sc-17320, RRID:AB_2286684
	Nanog (rabbit)	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		Thermo Fisher Scientific Cat# A-11017,
	Goat anti-Rabbit IgG Alexa Fluor 488	1:1000	RRID:AB_2534084
	Donkey anti-Rabbit IgG Alexa Fluor 568		Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165
	Rabbit anti-Goat IgG Alexa Fluor 568	1:1000	Thermo Fisher Scientific Cat# A10042, RRID:AB_2534017
		1:1000	
		1:1000	Thermo Fisher Scientific Cat# A-11079, RRID:AB_2534123

Primers		
	Target	Forward/reverse primer (5′-3′)
Episomal Plasmid (qPCR)	EBNA1	ATC AGG GCC AAG ACA TAG AGA TG
		GCC AAT GCA ACT TGG ACG TT
Pluripotency Markers (qPCR)	NANOG Hu F	CCT GTG ATT TGT GGG CCT G
	NANOG Hu F	GAC AGT CTC CGT GTG AGG CATAGC CAT ATG GTA GCC TCA TGT CCG C
		TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
		TTC ACA TGT CCC AGC ACT ACC AGA
	LIN28 Hu F	TCA CAT GTG TGA GAG GGG CAG TGT GC
	LIN28 Hu R	CCC CAG GGC CCC ATT TTG GTA CC
	SOX2 Hu F	ACC TCA GTT TGA ATG CAT GGG AGA GC
	SOX2 Hu F	
	OCT4 Hu F	
	OCT4 Hu R	
House-Keeping Genes (qPCR)	GAPDH Hu F	ACC ACA GTC CAT GCC ATC AC
	GAPDH Hu R	TCC ACC ACC CTG TTG CTG TA
Differentiation Markers (qPCR)	TubB3 Hu F	GCTCAGGGGCCTTTGGACATCTCTT
	TubB3 Hu R	TTTTCACACTCCTTCCGCACCACATC
		CGAGAGGACCCCGTGGATGCAGAG
	MSX1 Hu F	GGCGGCCATCTTCAGCTTCTCCAG
	MSX1 Hu R	GAATGCTGCAAACTGACCACGCTGGAAC
	AFP Hu F	TGGCATTCAAGAGGGTTTTCAGTCTGGA
	AFP Hu R	
Targeted mutation analysis/sequencing	HBB_ex0102 F2	TGTAAAACGACGGCCAGTAAGTCAGGGCAGAGCCATC
	HBB_ex0102 R	CAGGAAACAGCTATGACCACTGTACCCTGTTACTTATC

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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.2018.09.011.

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