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Lab Resources

Generation of human iPS cell line CBTCi001-A from dermal fibroblasts obtained from a healthy donor

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A B S T R A C T

Human-induced pluripotent stem cell (hiPSC) CBTCi001-A line was generated from a healthy 30-year old male dermal fibroblasts using non-integrative reprogramming method using episomal-based plasmids expressing OCT4, SOX2, KLF4, and MYCL. Characterization of CBTCi001-A was confirmed by the expression of typical markers of pluripotency and differentiation potential *in vitro*.

Resource Table:

Unique stem cell line identifier	CBTCi001-A
Alternative name(s) of stem cell line	EA1c2-MS
Institution	Center for Biotechnology and Cell Therapy - São Rafael Hospital
Contact information of distributor	Bruno Solano - bruno.solano@fiocruz.br
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 30
Sex: male	
Ethnicity: Caucasian	
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal vectors carrying OCT3/4, shRNA p53, SOX2, KLF4, LIN28, MYCL, and EBNA1
Genetic 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	04/04/2018
Cell line repository/bank	N/A
Ethical approval	CAAE: 20032313.6.0000.0048

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1. Resource utility

Integration-free human induced pluripotent stem cell (hiPSC) line from dermal fibroblasts of a healthy male individual previously used in Souza et al., 2016 and Nonaka et al., 2019 and can be used in a wide array of assays to test different differentiation protocols and as a control cell line for drug development and disease modeling studies.

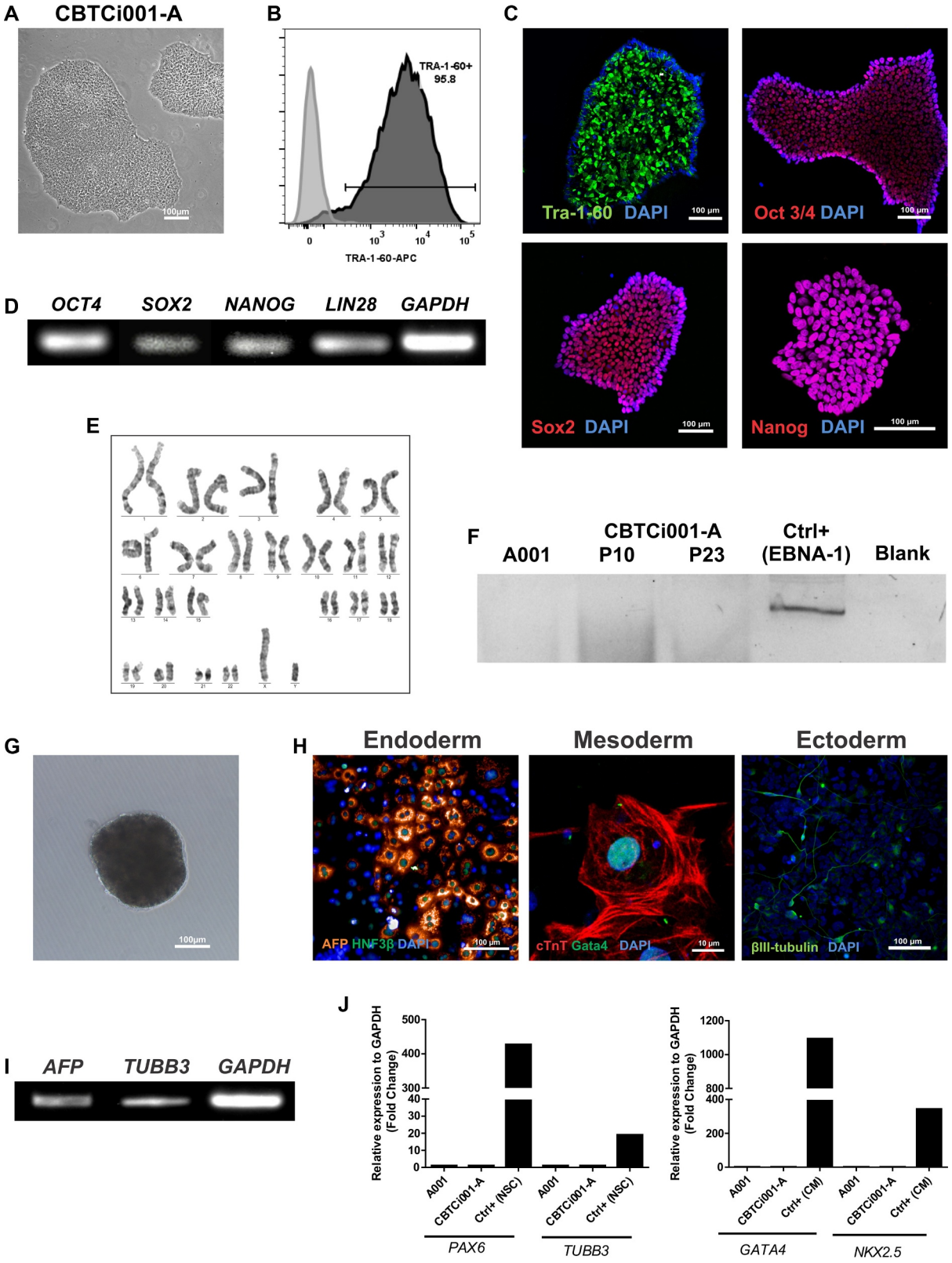

Fig. 1. Characterization panel of iPS cell line CBTCi001-A, obtained by dermal fibroblast reprogramming from a healthy donor.

2. Resource details

Dermal fibroblasts from a healthy donor were obtained from skin punch biopsy, according to approved institutional procedures. To generate the iPSCs, we delivered human OCT3/4, SOX2, KLF4, MYCL, LIN28, shRNA of TP53 and EBNA1 using episomal expression vectors (Okita et al., 2011) by nucleofection of fibroblasts at p4. 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 100 μm). The expression of endogenous pluripotency genes OCT4, SOX2, NANOG, LIN28 was detected by RT-PCR (Fig. 1D). Chromosomal stability was confirmed by G-band karyotyping analysis at passage 15 (Fig. 1E) showing no numerical or structural clonal alterations. The loss of the episomal vectors used for cell reprogramming was confirmed by PCR for EBNA1 at P10 and P23 (Fig. 1F). The iPS cells generated EBs presented the typical morphology (Fig. 1G, scale bar 100 μm). CBTCi001-A demonstrated the ability to generate derivatives of the three-germ layers in directed differentiation assays towards cardiomyocyte (cTnT and Gata4), hepatoblasts (AFP and HNF-3β) and neural progenitors (Tubulin-b3)(Fig. 1H, scale bar 100 μm). Gene expression of endoderm (AFP) and ectoderm (TUBB3) markers was also detected by RT-PCR (Fig. 1I). Additionally, the expression of ectoderm markers PAX6 and TUJ1, mesoderm markers GATA4 and NKX2.5 were accessed by qPCR. The cell line was tested for *Mycoplasma* sp., showing negative results. Finally, genetic fingerprinting was performed by STR analysis, confirming the genetic identity to the parental fibroblasts (available with the authors). Full characterization is summarized in Table 1.

3. Materials and methods

3.1. Ethics statement

Selection of human healthy donor, as well as the procedures for skin biopsies, isolation, and characterization of dermal fibroblasts, were done in accordance with protocols approved by the Ethics Committee of Hospital São Rafael. Informed consent was given before the start of the study.

3.2. Maintenance and reprogramming of skin fibroblasts

Dermal fibroblsts were obtained from a healthy donor skin biopsy and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in an incubator at 37 ºC humidified with 5% CO_2. At passage 4 (P4), dermal fibroblasts were trypsinized with trypsin-EDTA (ThermoFisher), resuspended in medium with FBS and centrifuged at 340 x g for 5 min. Cells were counted and 10^6 cells were prepared for reprogramming by nucleofection using 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) (Nonaka et al., 2019; Okita et al., 2011; Souza et al., 2016) using the Nucleofection kit P2 solution and Nucleofector 4D (Lonza), program DS-150. Transfected cells were plated in 3 wells of a 6-well plate pre-coated with Matrigel hESC- qualified (Corning) in DMEM + 10% FBS medium. After two days, the medium was changed to ReproTeSR media kit (Stemcell Technologies) and the protocol was performed as described in the manufacturer's instructions. Grown iPS cell colonies were picked in between days 15 and 25. Picked colonies were transferred to Matrigel pre-coated 24-wells plate with mTeSR1 medium. Criteria to select the best cell clones were based on colony morphology, growth pattern and intensity of TRA-1–60 staining in live staining assay (data not shown).

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

The 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 F12 supplemented with 20% KOSR, 1% Non-Essential Amino Acids, 1 × Glutamax, 1% Penicillin/Streptomycin (all from Thermo Fisher Scientific), FGF (4 ng/mL) (Gibco) and 10 μM Y-27,632 (Stemcell Technologies). Next, 1 mL of a 9 × 10^5 cells/mL cell suspension was transferred to an Aggrewell plate (StemCell Technologies) for EB formation, according to the manufacturer's instructions. Half of the media was replaced after two days. After one week, the aggregates were replated into a non-adherent 60 mm plate, where the cells were maintained for two weeks.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase-contrast microscopy image	PSC morphology confirmed	Fig. 1, panel A
Phenotype	Qualitative analysis Immunofluorescence and PCR	The iPSC express pluripotency markers (TRA 1-60, Sox2, Oct4 and Nanog) Endogenous pluripotency genes (*OCT4, NANOG, SOX-2* and *LIN28*)	Fig. 1, panel C (IF); Fig. 1, panel D (PCR)
	Quantitative analysis Flow cytometry	TRA-1-60 - 95.8%	Fig. 1, panel B
Genotype	Karyotype (G-banding) and resolution	46 XY	Fig. 1, panel E
Identity	Microsatellite PCR (mPCR) OR	N/A	N/A
	STR analysis	24 loci were analyzed and all matched with the parental cell line	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	Mycoalert (Lonza) – Negative	Supplement document
Differentiation potential	Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	Embryoid body; *in vitro* Directed differentiation in Neural Stem Cells (BIII Tubulin, Hepatic progenitors cells (HNF-3β and AFP), and Cardiomyocytes (GATA4 and cTnT) by IF; Gene expression of *AFP, TUBB3, PAX6, GATA4*, and *NKX2.5* by PCR	Fig. 1, panel G (EB) Fig. 1, panel H (IF) Fig. 1, panel I and J
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

4. Directed differentiation

4.1. Endoderm lineage differentiation

Endoderm lineage differentiation was performed by plating CBTCi001-A cells at a density of 7.5×10^4 cells/cm^2 per well of a 24-well plate and kept in mTeSR1 for 1 day. On the next day, the PSC Endoderm Differentiation medium kit (Thermo Fisher Scientific) was used according to the manufacturer's manual. Then, the medium was changed to RPMI 1640 with Glutamax (Gibco) supplemented with 20 ng/mL HGF (Peprotech) for 7 days, changing medium every other day. Differentiated cells were then fixed in formaldehyde 4% for immunofluorescence staining or collected for qPCR analysis.

4.2. Ectodermal lineage differentiation

For ectoderm lineage differentiation, we derived Neural Stem Cells (NSC) from CBTCi001-A by using the PSC Neural Induction kit (Thermo Fisher Scientific) according to the manufacturer's manual. Briefly, cells were plated in a low density (2.5×10^4 cells/cm^2) per well of a 6-well plate with mTeSR1 for 1 day. In the next two days, the medium was changed to PSC neural induction medium and change every other day until day 7. After two passages the NSC derived iPSC were fixed in formaldehyde 4% for immunofluorescence staining or collected for qPCR analysis.

4.3. Mesodermal differentiation

CBTCi001-A was differentiated to cardiomyocytes by using the PSC Cardiomyocyte differentiation kit (Thermo Fisher Scientific) according to the manufacturer's manual. Briefly, cells were plated at a high density (10^6 cells/cm^2) per well of a 12-well plate with mTeSR1 for 4 days. On the following day, the medium was changed to PSC Cardiomyocyte A medium for 2 days, followed by PSC Cardiomyocyte B medium for 2 days. Medium was changed to PSC Cardiomyocyte maintenance medium, with changes every other day. Beating cells could be observed after 10 days of differentiation protocol. Cardiomyocytes were fixed in formaldehyde 4% for immunofluorescence staining or collected for qPCR analysis.

4.4. Immunofluorescence assay

The iPS cells were grown on coverslips with Geltrex (Gibco) and fixed with formaldehyde 4% for 15 min and were washed twice with PBS 1X. To nuclear antibodies staining, cells were permeabilized with 0.1% Triton X-100 for 15 min and blocked with 5% bovine serum albumin (BSA) during 40 min at room temperature (RT). Pluripotency was assessed using primary antibodies TRA-1–60, Oct4, Sox2 and Nanog diluted in PBS BSA 0.1%. and then stained with appropriate secondary antibody diluted in PBS BSA 0.1% for 1 h at room temperature. Differentiation ability of iPS-directed differentiation cells was assessed by using primary antibodies to alpha-fetoprotein (AFP) and HNF-3β for endoderm lineage, cardiac troponin T (cTnT) and Gata-4 for mesoderm lineage, and tubulin βIII isoform (B3tub) for ectoderm lineage. All antibodies were diluted in PBS BSA 0.1% and incubated overnight and then washed with PBS Tween-20 0.05% and PBS 1X for 3 min each. The appropriate secondary antibodies were diluted in PBS BSA 0.1% and incubated for 1 h at room temperature. Cells were washed twice with PBS Tween-20 0.05% and PBS 1X for 3 min. The slides were mounted using Vectashield with DAPI for nuclei staining. Images were acquired with Nikon A1 confocal microscope.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	TRA 1–60 (mouse)	1:250	Abcam Cat# ab16288, RRID: AB_778563
	Oct4 (rabbit)	1: 500	Abcam Cat# ab181557, RRID: AB_2687916
	Sox2 (rabbit)	1:500	Cell signaling Cat# D9B8N, RRID: AB_2714146
	Nanog (rabbit)	1:125	Abcam Cat# ab80892 AB_2150114
Differentiation Markers	Tubulin beta III isoform (mouse)	1:200	Millipore Cat# MAB1637, RRID: AB_2210524)
	AFP (rabbit)	1:300	Innovative Research Cat# 18-0055, RRID: AB_138884
	HNF-3β (goat)	1:200	Santa Cruz Biotechnology Cat# sc-6554, RRID: AB_2262810
	cTnT (mouse)	1:200	Thermo Fisher Scientific Cat# MA5-12960, RRID: AB_11000742
	Gata-4 (rabbit)	1:250	Santa Cruz Biotechnology Cat# sc-9053, RRID:AB_2247396
Secondary antibodies	Goat anti-Mouse IgM Alexa fluor 488	1:800	Thermo Fisher Scientific Cat# A-21042, RRID:AB_2535711
	Goat anti-Rabbit IgG Alexa fluor 568	1:1000	Thermo Fisher Scientific Cat# A-11011, RRID:AB_143157
	Goat anti-Rabbit IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165
	Chicken anti-Mouse IgG Alexa fluor 488	1:1000	Thermo Fisher Scientific Cat# A-21200, RRID:AB_2535786
	Donkey anti-Mouse IgG Alexa Fluor 647	1:1000	Thermo Fisher Scientific Cat# A32787, RRID:AB_2762830)
	Donkey anti-Goat IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A32814, RRID:AB_2762838
Primers			
	Target	Forward/Reverse primer (5′ − 3′)	
Episomal plasmid	*EBNA1*	F: GGT TTT GAA GGA TGC GAT TAA G R: TTT AAT ACG ATT GAG GGC GTC T	
Pluripotency Markers	*NANOG* (78 bp)	Forward: CCT GTG ATT TGT GGG CCT G Reverse: GAC AGT CTC CGT GTG AGG CAT	
	LIN28 (129 bp)	Forward: AGC CAT ATG GTA GCC TCA TGT CCG C Reverse: TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG	
	SOX-2 (80 bp)	Forward: TTC ACA TGT CCC AGC ACT ACC AGA Reverse: TCA CAT GTG TGA GAG GGG CAG TGT GC	
	OCT4 (143 bp)	Forward: CCC CAG GGC CCC ATT TTG GTA CC Reverse: ACC TCA GTT TGA ATG CAT GGG AGA GC	
House-Keeping Genes	*GAPDH* (197 bp)	Forward: GGA GCG AGA TCC CTC CAA AAT Reverse: GGC TGT CAT ACT TCT CAT GG	
Differentiation Markers	*AFP* (174 bp)	Forward: ACT GAA TCC AGA ACA CTG CA	
	TUBB3 (148 bp)	Reverse: TGC AGT CAA TGC ATC TTT CA	
		Forward: GCT CAG GGG CCT TTG GAC ATC TCT TT	
		Reverse: TTT TCA CAC TCC TTC CGC ACC ACA TC	
e.g. Targeted mutation analysis/sequencing	N/A	N/A	

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

Total RNA was extracted from iPSC cells 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, TUBB3, EBNA-1*, and the housekeeping gene *GAPDH*, using primers described in Table 2. PCR products were visualized by electrophoresis on agarose gel 2%. For RT-qPCR analysis, TaqMan® Universal PCR Master mix and TaqMan® Assays were used: *PAX6* (Hs01088114_m1), *TUBB3* (Hs00801390_s1), *GATA4* (Hs00171403_m1) and *NKX2-5* (Hs00231763_m1), according to the manufacturer's instructions (all ThermoFisher Scientific). Signals were detected by ABI7500 Fast (Applied Biosystem). All data were normalized against *GAPDH* (Hs04420632_g1) mRNA levels using 2-ΔΔCt method and graphic representation by GraphPad Prism 7.0.

4.6. Short tandem repeat (STR) analysis

STR analyses were performed by pairing the parental cell source (skin fibroblasts) of the 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).

4.7. Karyotype analysis

The iPSCs were analyzed for chromosomal alterations by G-band karyotyping at P7. The cells were incubated with colcemid (0.1 μg/mL) for 1 h at 37 °C (5% CO2, 95% rH). The cells were then 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 was conducted in 20 metaphases using Lucia Karyo software (Lucia Cytogenetics) with a 400–450 band resolution.

4.8. Mycoplasma testing

Mycoplasma contamination test was performed by collecting 1 mL of iPS cell culture supernatant without antibiotics for 24 h and processed for use in MycoAlert Detection kit (LONZA), according to manufacturer's protocol.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101630.

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