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Lab resource: Stem Cell Line

Generation of induced pluripotent stem cells (iPSCs) from patient with Cri du Chat Syndrome

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A B S T R A C T

The Cri du Chat Syndrome (CdCS) is a genetic disease resulting from variable size deletion occurring on the short arm of chromosome 5. The main clinical features are a high-pitched monochromatic cry, microcephaly, severe psychomotor and mental retardation with characteristics of autism spectrum disorders such as hand flapping, obsessive attachments to objects, twirling objects, repetitive movements, and rocking. We reprogrammed to pluripotency peripheral blood mononuclear cells derived from a patient carrying large deletion on the short arm of chromosome 5, using a commercially available non-integrating expression system. The iPSCs expressed pluripotency markers and differentiated in the three embryonic germ layers.

Resource table

Unique stem cell line identifier	UNIBSi004-A
Alternative name(s) of stem cell line	*BF17–1*
Institution	Department of Molecular and Translational Medicine, University of Brescia, 25,123 Brescia, Italy
Contact information of distributor	Giovanna Piovani: giovanna.piovani@unibs.it
Type of cell line	iPSCs
Origin	Human
Additional origin info	Age: 35 Sex: male Caucasian
Cell source	Blood
Clonality	Clonal
Method of reprogramming	CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific). The episomal reprogramming vectors include the four Yamanaka factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc*
Genetic Modification	NO
Type of Modification	N/A
Associated disease	Cri du Chat Syndrome
Gene/locus	del(5)(p14)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	Feb 2018
Cell line repository/bank	N/A
Ethical approval	The study was approved by the Scientific Committee and by the Board of the ABC. Written informed consent was obtained from patient's parent.

Resource utility

We generated a human cellular Cri du Chat model starting from a CdC patient carrying large deletion in the short arm of one chromosome 5. We believe that iPSCs will be fundamental to derive otherwise unavailable neurons with the same genetic background, offering a tool for studying this genetic disease.

Resource details

Cri du Chat Syndrome (CdCS) is a genetic disorder caused either by a partial or complete deletion of the short arm of chromosome 5. The incidence ranges from 1:15,000 to 1:50,000 live born infants. Most of the deletions occur *de novo*, and are mostly (80–90%) of paternal origin, which can arise from a chromosomal breakage during gamete formation, while the minority are due to an unbalanced parental translocation (Cerruti Mainardi, 2006; Cornish and Pigram, 1996; Ajitkumar and Whitten, 2018). Although the classic 5p–phenotype include a characteristic cry, dysmorphic features, growth, and developmental delay,

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there is a wide spectrum of features, depending on the region of the chromosome that is deleted and whether it is terminal or interstitial (Nguyen et al., 2015). In this study we generated patient-specific iPSC line, UNIBSi004-A, from the peripheral blood mononuclear cells (PBMCs) of a 35-year-old male affected by CdCS. The patient's karyotype shows a large deletion, from band p14 to the end of the short arm of one chromosome 5 (46,XY,del(5)(p14)). The patient shows typical clinical features of CdCS as dysmorphic traits, severe psychomotor and mental retardation and characteristics of autism spectrum disorders. The PBMCs were reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit, in feeder free condition. This kit utilizes a non-transmissible, non-integrating form of Sendai virus (SeV) that carries the Yamanaka's factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. After approximately 20 days from SeV infection, we manually picked and expanded several single cell-derived iPSCs colonies. Among those, one sub-clone that best displayed ESC-like morphology (Fig. 1A) was stabilized and characterized. We assessed that UNIBSi004-A cell line was mycoplasma-free (Supplementary Fig.1) and we analyzed the karyotype at different culture passages (p6, p21, and p33), confirming the cell line original 46,XY,del(5)(p14) karyotype (Fig. 1B the arrow shows the deleted chromosome). STR analysis that uniquely identify UNIBSi004-A iPSCs has been reported in Table 1. Next, we analyzed the expression of the endogenous pluripotent transcription factors and the transgene-free status of the iPSC line at passage 21 by quantitative PCR, using the primers reported in Table 2. We confirmed the expression of endogenous *NANOG*, *OCT4*, *SOX2*, *c-MYC*, and *KLF4* fully comparable to those of a commercial certificate control iPSC line (CTL-hiPSC, Gibco® Episomal hiPSC Line, Cat#A18945); moreover, the absence of transgene expression confirmed the clearance of the viral vectors, as shown in Fig. 1C. The presence of two stem cell markers, Tra-1-60 and OCT4, was also assessed by immunofluorescence staining using the antibodies listed in Table 2. The images obtained show how the Tra-1-60 marker is properly present on cell surface, while the transcriptional factor OCT4 is expressed at nuclear level (Fig. 1D).

Pluripotent UNIBSi004-A iPSC line at passage 33, was finally investigated for spontaneous differentiation capacity in the three germ layers by the expression of ectodermal, mesodermal and endodermal markers (*PAX6-SOX1*, *NCAM1-ACTA2*, *GATA4-SOX17*, respectively) (Table 2 and Fig.1E).

In conclusion, we generated an iPSC line carrying the 5p14 deletion. Potentially, the study of the meaning of the heterozygous state of the genes inside the deletion will strongly benefit by UNIBSi004-A iPSC-derived neuron analysis, to understand the syndrome etiopathology.

Materials and methods

Reprogramming of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by Lympholyte®–H Cell Separation Media (Cedarlane). Before reprogramming, PBMCs were cultured for 4 days in StemPro®-34 SFM Medium (ThermoFisher Scientific) with SCF, FLT-3 (100 ng/ml), IL-3, and IL-6 (20 ng/ml) (Gibco) cytokines. Then, PBMCs were transduced using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific) following manufacturer's instructions. After 20 days, colonies positive to Tra1–60 staining were manually picked and expanded for further characterization. iPSCs were cultured on matrigel-coated plates with daily renewal of the Nutristem hPSC XF medium (Biological-Industries).

iPSC karyotyping

For karyotyping, iPSCs undergoing active cell division were blocked at metaphase by 10 µg/ml of colcemid (Karyo Max, Gibco Co. BRL), detached by trypsin–EDTA, and subsequently swollen by exposure to hypotonic KCL (0,075 M) solution. The cells were fixed with methanol/glacial acetic acid (3:1) three times, and dropped onto glass slides.

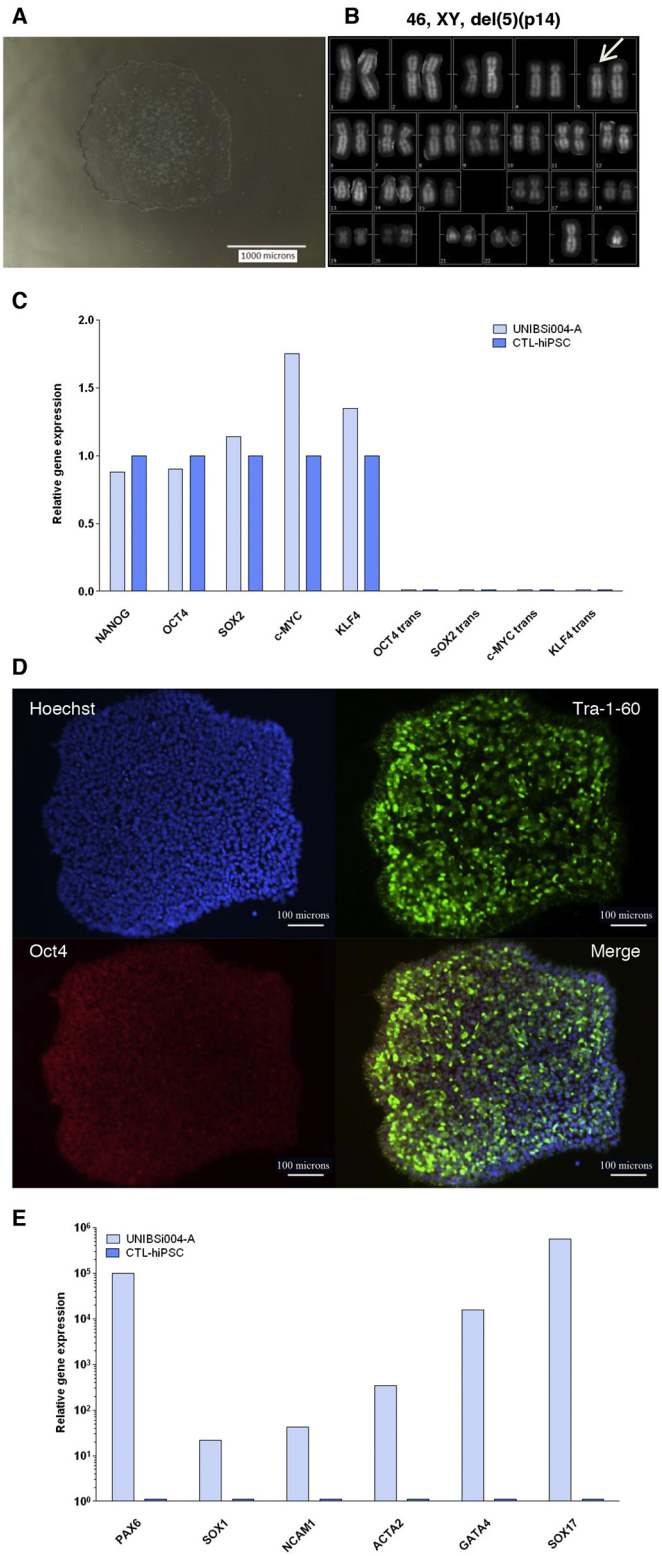

Fig. 1. Characterization of UNIBSi004-A iPSC line.

Cytogenetic analysis were performed using QFQ-banding at 450 bands resolution according to the International System for Human Cytogenetic Nomenclature (ISCN 2016). A minimum of 20 metaphase spreads and 3 karyograms were analyzed.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Fig. 1 panel A
Phenotype	Immunocytochemistry	Assess staining/expression of markers: OCT4, and TRA-1-60	Fig. 1 panel D
	RT-qPCR	Relative gene expression: *NANOG, OCT4, SOX2, c-MYC, KLF4,* and Cytotune-iPS 2.0 KOSM transgenes	Fig. 1 panel C
Genotype	Karyotype (Q-banding) and resolution	46,XY,del(5)(p14) Resolution 450–500	Fig. 1 panel B
Identity	Microsatellite PCR (mPCR) OR	*N/A*	*N/A*
	STR analysis	16 distinct loci: all matched to parental cell line	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	*N/A*	*N/A*
	Southern Blot OR WGS	*N/A*	*N/A*
Microbiology and virology	Mycoplasma	Negative	Supplementary Fig. 1
Differentiation potential	Trilineage Differentiation	Genes expressed in the *in vitro* differentiate three germ layers (Ectoderm: *PAX6-SOX1*; Endoderm: *GATA4-SOX17*; Mesoderm: *ACTA2-NCAM1*).	Fig. 1 panel E
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*

In vitro trilineage differentiation

Lineage-specific differentiation was performed using StemMACS Trilineage Differentiation Kit protocol (Miltenyi Biotec). Briefly, iPSCs were dissociated into single-cell and seeded on matrigel-coated 24-well plates (100,000, 80,000, 130,000 cells for ectoderm, mesoderm, and endoderm, respectively) in the specific medium. From day 1 to 6, media changes were performed according to the manufacturer's protocol. On day 7, cells were collected for total RNA extraction and quantitative PCR (qPCR) of lineage specific gene expression.

RNA extraction and qPCR analysis

Total RNAs were extracted using NucleoSpin® RNA II kit (Macherey-Nagel) and quantified by a Spectrofluorometer. Whenever request by the gene expression analysis conditions, a TURBO-DNase (Ambion) treatment was performed on RNA following the manufacturer instructions, to ensure the complete elimination of gDNA. RNAs were retro-transcribed by ImProm-II™ Reverse Transcription System (Promega), following the protocol. qPCR was assessed for pluripotency using iTaq™ Universal SYBR® Green Supermix, while three layers differentiation

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:400	Thermo Fisher Scientific, Cat# A-13998. RIID: AB_2534182
Pluripotency Markers	Mouse anti-TRA-1-60	1:100	Thermo Fisher Scientific, Cat# 4110000. RIID: AB_2533494
Secondary antibodies	Goat anti rabbit IgG (H + L) Alexa Fluor 568	1:300	Thermo Fisher Scientific, Cat# A-11011. RIID: AB_143157
Secondary antibodies	Goat anti mouse IgG (H + L) Alexa Fluor 488	1:300	Thermo Fisher Scientific, Cat# A-11001. RIID: AB_2534069

Pluripotency Primers for RT-qPCR with SYBR Green chemistry

	Target	Forward/Reverse primer (5′-3′)
Pluripotency Markers	*NANOG*	TGAACCTCAGCTACAAACAG/TGGTGGTAGGAAGAGTAAAG
Pluripotency Markers	*OCT4*	CCTCACTTCACTGCACTGTA/CAGGTTTTCTTTCCCTAGCT
Pluripotency Markers	*SOX2*	CCCAGCAGACTTCACATGT/CCTCCCATTTCCCTCGTTTT
Pluripotency Markers	*C-MYC*	TGCCTCAAATTGGACTTTGG/GATTGAAATTCTGTGTAACTGC
Pluripotency Markers	*KLF4*	GATGAACTGACCAGGCACTA/GTGGGTCATATCCACTGTCT
Sendai virus detection	*OCT4 transgene*	CCTCACTTCACTGCACTGTA/CCTTGAGGTACCAGAGATCT
Sendai virus detection	*SOX2 transgene*	CCCAGCAGACTTCACATGT/CCTTGAGGTACCAGAGATCT
Sendai virus detection	*C-MYC transgene*	TGCCTCAAATTGGACTTTGG/CGCTCGAGGTTAACGAATT
Sendai virus detection	*KLF4 transgene*	GATGAACTGACCAGGCACTA/CCTTGAGGTACCAGAGATCT
House-Keeping Genes	*βACTIN*	CGCCGCCAGCTCACCATG/CACGATGGAGGGGAAGACGG
Mycoplasma detection	*16S rRNA*	GGGAGCAAACAGGATTAGATACCCT/TGCACCATCTGTCACTCTGTTAACCTC

Differentiation RT-qPCR assays with TaqMan chemistry

	Target	Probe
Ectoderm	*PAX6*	Hs.PT.58.25914558
	SOX1	Hs.PT.58.28041414.g
Mesoderm	*ACTA2*	Hs.PT.56a.2542642
	NCAM1	Hs.PT.58.39694135
Endoderm	*GATA4*	Hs.PT.58.259457
	SOX17	Hs.PT.58.24876513
Housekeeping gene	*ACTB*	Hs.PT.39a.22214847

capacity was performed using iQ MPLX powermix and TaqMan Probe based assays. Primers and probes are listed in Table 2. Assays were performed on CFX96 C1000 Touch™ Real-Time PCR Detection System, and analyzed with CFX manager software v.3.1 (Bio-Rad). The relative quantification of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method, using *βACTIN* as housekeeping gene.

Immunofluorescence staining

Immunofluorescence was performed to verify the expression of specific markers. The antibodies used are summarized in Table 2. Cells were fixed 15 min with Reagent A and permeabilized with Reagent B (Fix&Perm-Reagent, *SIC*) at room temperature (RT). Then, a blocking solution iBindTM Buffer (Invitrogen) was applied for 45 min. The primary antibodieswere added and incubated for 3 h at RT. After washing, secondary antibodies were added 1 h at RT. Cellular nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific). Cells were observed with an inverted fluorescence microscope (Olympus IX70), and images were analyzed with the Image-Pro Plus software v7.0 (Media Cybernetics).

Mycoplasma test

Mycoplasma contamination was verified by PCR using the primers in Table 2.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101393.

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