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Derivation of induced pluripotent stem cells (iPSCs) by retroviral transduction of skin fibroblasts from four patients suffering 7q11.23 microduplication syndrome

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A B S T R A C T

Skin fibroblasts were obtained from four patients with 7q11.23 microduplication syndrome carrying the reciprocal rearrangement of Williams-Beuren syndrome at the 7q11.23 genomic region. Induced pluripotent stem cells (iPSCs) were generated by retroviral infection of fibroblasts with polycystronic vectors. The generated iPSC clones ESi058B, ESi057B, ESi070A and ESi071A had the 7q11.23 duplication with no additional genomic alterations, a stable karyotype, expressed pluripotency markers and could differentiate towards the three germ layers *in vitro* via embryoid body formation and *in vivo* by teratoma formation. Patient's derived iPSCs are a valuable resource for *in vitro* modeling of 7q11.23 microduplication syndrome.

Resource Table:

Unique stem cell lines identifier	ESi058B ESi057B ESi070A ESi071A
Alternative names of stem cell lines	DUP7 FiPS4 R4F-2, 4.2 (ESi058B) DUPSW FiPS301 R4F-1, 301.1 (ESi057B) DUPSW FiPS501 R4F-2, 501.2 (ESi070A) DUPSW FiPS701 R4F-6, 701.6 (ESi071A)
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Type of cell lines	iPSC
Origin	Human (Table 1)
Cell Source	Skin fibroblasts
Method of reprogramming	Retrovirus (*OCT4, SOX2, KLF4, MYC*)
Multiline rationale	Same disease, different patients
Gene modification	Yes
Type of modification	De novo formation
Associated disease	7q11.23 microduplication syndrome, ORPHA 96121
Gene/locus	7q11.23 duplication [hg19]7q11.23(72.7–74.2 Mb)x3
Method of modification	Duplication
Name of transgene or resistance	Not Applicable
Date archived/stock date	10.03.2017; 05.04.2018; 12.06.2018
Cell line repository/ bank	https://eng.isciii.es/eng.isciii.es/QueHacemos/Servicios/BIOBANCOS/BNLC/Paginas/LineasiPS.html https://hpscreg.eu/search?q = DUPSW
Ethical approval	Patient's parents informed consent obtained/ Ethics Review Board-competent authority approval obtained (Comité de Ética e Investigación Clínica-CEIC-CMRB) and by the Catalan Authority for Stem Cell Research (Approval number: 05/2011)

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1. Resource utility

7q11.23 microduplication syndrome (DUPSW) is a multisystemic neurodevelopmental disorder caused by ~ 1.5–1.8 Mb duplications at chromosome 7q11.23, encompassing 26–28 genes (Somerville et al., 2005). Patients present speech disorders, behavioral problems and developmental delay, among others. The availability of iPSCs facilitates the study of functional mechanisms, as those occurring during development or in inaccessible tissues.

2. Resource details

The iPS lines were generated from fibroblasts derived from skin biopsies obtained from patients suffering 7q11.23 microduplication syndrome, the reciprocal rearrangement of Williams-Beuren Syndrome. Three patients carry the typical 1.5 Mb hemizygous duplication at chromosome 7q11.23 (Table 1) and DUPSW FiPS501 R4F-2 carries a 2 Mb duplication. DUPSW is a rare genetic neurodevelopmental disorder [ORPHA 96121] caused by a heterozygous duplication of multiple contiguous genes at 7q11.23 (Somerville et al., 2005). The clinical characteristics of the patients include, intellectual disability, macrocephaly, dysmorphology, bilateral hearing loss and labial palate cleft (DUPSW FiPS301 R4F-1); intellectual disability, language delay, anxiety, attention-deficit/hyperactivity disorder (ADHD) and obsessive compulsive symptomatology, mitochondrial cytopathy, nistagmus, and mild tricuspid insufficiency (DUP7 FiPS4 R4F-2); language and developmental delay, behavioral problems (including autism spectrum disorders (ASD)), cryptorchidism, bilateral rectum paresis, constipation, dentation alterations, in addition to congenital cataracts and discrete tetraventricular hydrocephaly (DUPSW FiPS501 R4F-2); language and mild developmental delay with learning difficulties and behavioral problems within the board ASD spectrum, ADHD, anxiety, and occasional aggression (DUPSW FiPS701 R4F-6). The duplications had occurred *de novo* in the maternal (DUPSW FiPS301 R4F-1, DUPSW FiPS701 R4F-6) or paternal (DUP7 FiPS4 R4F-2, DUPSW FiPS501 R4F-2) chromosomes and were confirmed by molecular karyotype (Fig. 1A), MLPA and microsatellite analysis (Bayés et al., 2003). Except for DUPSW FiPS501 R4F-2, the origin of these rearrangements was probably mediated by the presence of a paracentric inversion in one of the parental chromosomes. Table 2.

Skin derived fibroblasts were reprogrammed using two polycystronic retroviruses encoding the four factors (pMXs-OCT4-VP16-SOX2-mOrange; pMXs-KLF4-MYC-GFP). The morphology of the colonies was the typical of iPSCs (Fig. 1B). Integration of retroviruses (Supplementary Fig. S1B) and silencing of retroviruses (Supplementary Fig. S1C) were determined by PCR and quantitative RT-PCR (qRT-PCR) using specific primers (Table 3). The pluripotency of the DUPSW iPSC lines was confirmed by immunofluorescence analysis of the pluripotency-associated markers OCT4, SOX2, NANOG, TRA-1–60, TRA-1–81, SSEA-3 and SSEA-4 (Fig. 1B, scale bars 50 μm), the mRNA expression of endogenous human factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* in comparison to control iPSC lines (hPSCreg: ESi045D and ESi045D), (Fig. 1C) and alkaline phosphatase (AP) activity (Supplementary Fig. S1E). The capacity of *in vitro* and *in vivo* differentiation towards the three germ layers was determined by embryoid body (EB) formation and differentiation and teratoma formation, respectively, followed by immunofluorescence-based detection of the definite endoderm markers α-fetoprotein (AFP) and forkhead box A2 (FOXA2), ectodermal markers βIII-tubulin (TUJ1), glial fibrillary acidic protein (GFAP) and neurofilament, and mesodermal markers α-smooth muscle actin (ASMA) and α-sarcomeric actin (ASA) (Fig. 1D and 1E, scale bars 50 μm). The normal 46, XX or 46, XY karyotypes of the iPSC lines were confirmed by G-banding analysis (Supplementary Fig. S1A). The genomic integrity of the iPSC lines and presence of the 1.5 or 2 Mb 7q11.23 duplication was confirmed by SNP-array (Fig. 1A) and the iPSC identity was confirmed by short tandem repeat analysis and compared with the patient́s fibroblasts (Supplementary Table S1).

3. Materials and methods

3.1. Reprogramming and cell culture conditions

Skin derived fibroblasts at passage 4 were used for reprogramming by retroviral delivery. Retroviruses were produced in Phoenix Amphotrophic cells following transfection with pMX-OCT4_Flag-VP16-PTV-Sox2_HA-Orange or pMX-KLF4-cMYC-GFP polycistronic vectors. Retrovirus containing medium was collected 48 h post-transfection, filtered (0.45 μm), supplemented with Polybrene (4 μg/ml) and used to transduce $1x10^5$ cells by spin infection. After 3 days, transduced fibroblasts were trypsinized, seeded onto irradiated human foreskin fibroblasts in HES medium (Knockout DMEM supplemented with 20% Knockout serum replacement, 2 mM Glutamax, 1% Penicillin-Streptomycin (P-S), 0.1 mM β-mercaptoethanol, Gibco), 1% NEAA and 10 ng/ml bFGF (Millipore)) and incubated in a humified incubator at 37 °C and 5% CO_2. Appearing iPSC colonies were manually picked for expansion. From passage 5 on, clones were changed to feeder-free conditions on matrigel (Corning) coated plates and mTSeR1 medium (StemCell Technologies). Passages were performed by incubating iPSC colonies with 0.5 mM EDTA for 2 min and cell clumps were generated by flushing colonies with a P1000 automatic pipette.

3.2. PCR and quantitative RT-PCR

For integration analysis, genomic iPSC DNA was isolated (Qiagen). Standard PCR was performed using BioTaq Polymerase (Table 3) and visualized in 1.5% agarose gel, using fibroblasts as negative control.

For silencing analysis, total RNA was isolated following standard Trizol-based procedure. 1 μg of RNA was used for reverse transcription (SuperScript-II, Thermo-Fisher Scientific). The qRT-PCR was performed using Power SYBR Green PCR Master Mix in an ABIPrism 7900 thermocycler (Applied Biosystems). Ct values were normalized with *GAPDH* as housekeeping gene and data analyzed with 2-ΔΔCt method. mRNA expression levels of virus derived transOCT4/SOX2 and transKLF4/cMyc and endogenous pluripotency markers *OCT4, SOX2, KLF4* and c-*MYC* were analyzed (Supplementary Fig. S1C and Fig. 1C).

3.3. Karyotyping and AP staining

Genomic integrity was confirmed by G-banded metaphase karyotype (Ambar, Barcelona). Briefly, 70% confluent feeder-free iPSC colonies were treated with colcemid (KaryoMax, Invitrogen), trypsinized,

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
DUP7 FiPS4 R4F-2 (ESi058B)	4.2	Female	13	Caucasian	Duplication	7q11.23 microduplication syndrome
DUPSW FiPS301 R4F-1 (ESi057B)	301.1	Female	15	Caucasian	Duplication	7q11.23 microduplication syndrome
DUPSW FiPS501 R4F-2 (ESi070A)	501.2	Male	17	Caucasian	Duplication	7q11.23 microduplication syndrome
DUPSW FiPS701 R4F-6 (ESi071A)	701.6	Male	4	Caucasian	Duplication	7q11.23 microduplication syndrome

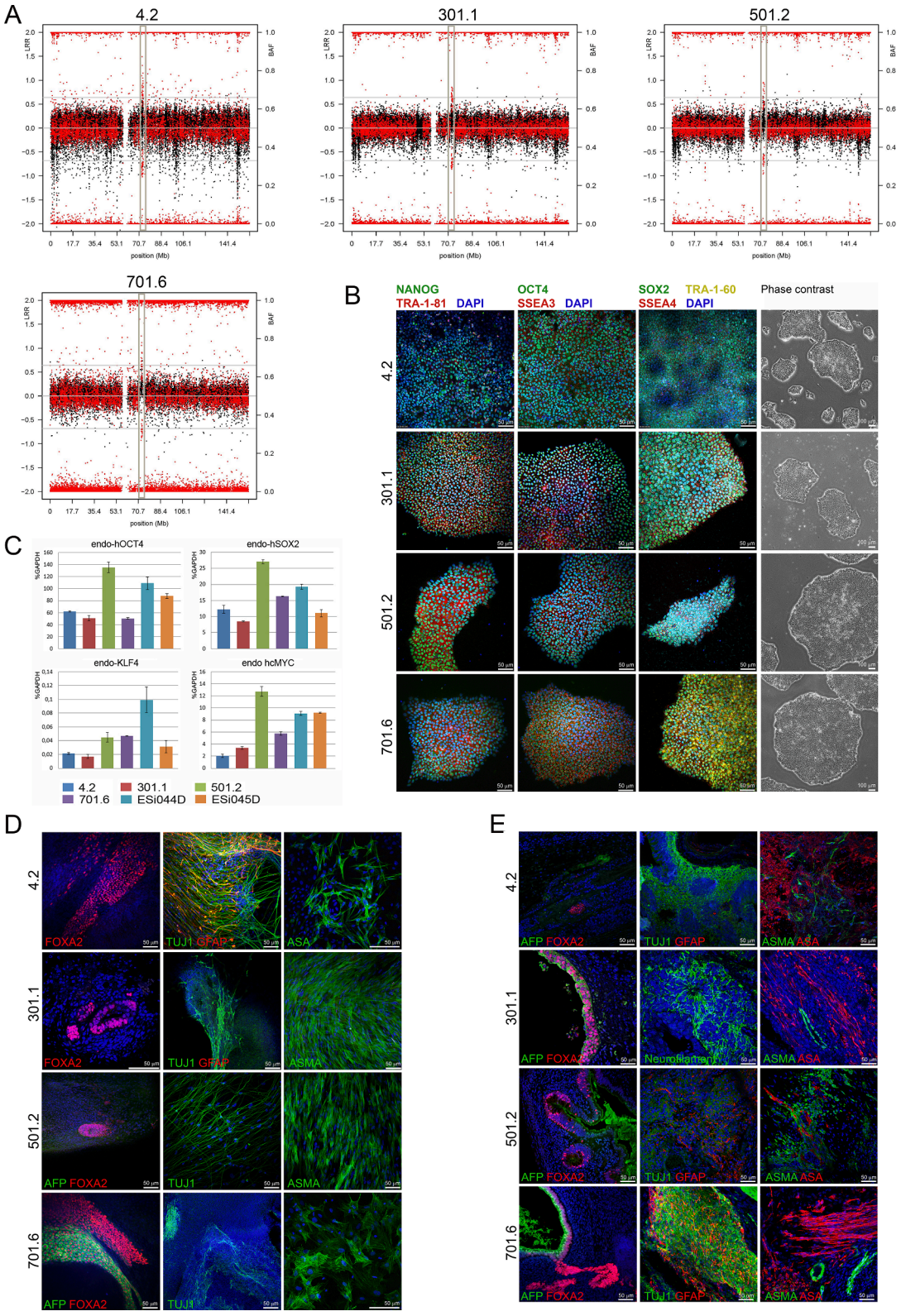

Fig.1. Characterization of DUPSW iPSC lines. A. SNP-arrays of lines showing the presence of the 1.5 Mb 7q11.23 duplication. B. Confocal images showing immunodetection of pluripotency markers. Scale bars: 50 μm. Phase contrast pictures (right column, scale bars: 100 μm) showing morphology of iPSC clones. C. mRNA expression levels of endogenous pluripotency markers in comparison to control iPSC lines ESi044-D and ESi045-D. 2-ΔΔCt values normalized to GAPDH. D. *In vitro* differentiation of embryoid bodies using specific antibodies against the endodermal markers α-fetoprotein (AFP) and forkhead box A2 (FOXA2), ectodermal markers βIII-tubulin (TUJ1) and Glial fibrillary acidic protein (GFAP) and mesodermal marker α-smooth muscle actin (ASMA) and α-sarcomeric actin (ASA). Nuclei were stained with DAPI. Scale bars: 50 μm. E. *In vivo* differentiation and teratoma formation using specific antibodies against the endodermal markers AFP and FOXA2, ectodermal markers TUJ1, GFAP and Neurofilament and mesodermal marker ASMA and ASA. Nuclei were stained with DAPI. Scale bars: 50 μm.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Phenotype	Immunocytochemisty	OCT4, SOX2, NANOG, TRA-1–60, TRA-1–81, SSEA-3 and SSEA-4	Fig. 1 panel B
	Gene expression (qRT-PCR)	*endo*-hOCT4, *endo*-hSOX2, *endo*-KLF4, *endo*-c-MYC, *trans*-OCT4/SOX2, *trans*-KLF4/c-MYC	Fig. 1 panel C Supplementary Fig. S1 panel C
	Integration PCR	Integration of retroviral OCT4, SOX2, KLF4 and c-MYC	Supplementary Fig. S1 panel B
	AP staining	positive	Supplementary Fig. S1 panel E
Genotype	Karyotype (G-banding) and resolution	ESi058B, 46XX, resolution 500 ESi057B, 46XX, resolution 500 ESi070A, 46XY, resolution 500 ESi071A, 46XY, resolution 500	Supplementary Fig. S1 panel A
Identity	STR analysis (microsatellite Study)	Performed (10 markers tested, all matching)	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Molecular Karyotype	Molecular karyoype Illumina Infinium Human Core-24 BeadChip, IMPPC or CEGEN	Fig. 1 panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. S1 panel D
Differentiation potential	Embryoid body formation and Teratoma formation	(1) Embryoid body formation: AFP, FOXA2, TUJ1, GFAP, ASMA, ASA (2) Teratoma formation: AFP, FOXA2, Neurofilament, TUJ1, GFAP, ASMA, ASA	Fig. 1 panel D Fig. 1 panel E
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)	N/A	N/A	N/A

incubated with hypotonic solution and fixed in Carnoy fixative (75% methanol, 25% acetic acid).

To detect AP activity, iPSCs were fixed with 4% paraformaldehyde (PFA) for 1 min, PBS washed and incubated with AP staining solution (Sigma) until colonies turned blue.

3.4. Immunocytological staining

To identify pluripotency markers, immunocytochemistry was performed (Martí et al., 2013). iPSCs were fixed with 4% PFA, blocked and permeabilized with TBS + 0.5% Triton X-100 + 6% donkey serum. Primary antibodies (Table 3) were incubated overnight in TBS + 0.1% Triton X-100 + 6% donkey serum. Secondary antibodies (Table 3) were incubated for 2 h at 37 °C. Nuclei were stained with 4′,6-diamino-2-

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4	1:25	Santa Cruz, sc-5279, RRID:AB_628051
	Goat anti-NANOG	1:25	R&D Systems, AF1997, RRID:AB_355097
	Rabbit anti-SOX2	0.1111	ABR, PA1-16968, RRID: AB_2195781
	Rat anti-SSEA3	1:02	Hybridoma Bank, MC-631, RRID:AB_528476
	Mouse anti-SSEA4	1:02	Hybridoma Bank, MC-813–70,RRID: AB_528477
	Mouse anti-TRA-1–60	0.1111	Millipore, MAB4360, RRID:AB_2119183
	Mouse anti-TRA-1–81	0.1111	Millipore, MAB4381, RRID:AB_177638
Differentiation Markers	Mouse anti-TUJ1	1:40	Covance, MMS-435P, RRID:AB_2313773
	Rabbit anti-GFAP	0.7361	Dako, Z0334, RRID: AB_10013382
	Rabbit anti-Neurofilament	0.1111	Sigma N4142, RRID: AB_477272
	Mouse anti-ASMA	0.3194	Sigma, A5228, RRID: AB_262054
	Mouse anti-ASA	0.3194	Sigma, A2172, RRID: AB_476695
	Rabbit anti-AFP	0.1806	Agilent, A0008, RRID: AB_2650473
	Goat anti-FOXA2	1:50	R&D Systems, AF2400, RRID:AB_2294104
Secondary antibodies	488 Donkey Anti-Mouse	0.1806	Jackson, 715–545-151, RRID:AB_2341099
	488 Goat Anti-Mouse	0.1806	Jackson, 115–546-071, RRID:AB_2338865
	488 Donkey Anti-Rabbit	0.1806	Jackson, 711–545-152, RRID:AB_2313584
	405 AffiniPure Donkey Anti-Rabbit	0.1806	Jackson, 711–475-152, RRID:AB_2340616
	Cy™3 Donkey Anti-Mouse	0.1806	Jackson, 715–165-140, RRID:AB_2340812
	Cy™3 Donkey Anti-Goat	0.1806	Jackson, 705–165-147, RRID:AB_2307351
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Integration	pMXs-OS-Orange	GAGCAAGGGCGAGGAGAATAAC/ AAGTAGTCGGGGATGTCGGC	
	pMX-KM-GFP	GCACCATCTTCTTCAAGGACGAC/ TCTTTCGCTCAGGGCGGACTG	
Silencing	Tg-mKLF4	CCACCTTGCCTTACACATGA/ CCTACAGGTGGGGTCTTTCA	
	Tg-mcMYC	GCTTCGAAACTCTGGTGCAT/ CCTACAGGTGGGGTCTTTCA	
	Tg-mSOX2	GGCCATTAACGGCACACT/ CCTACAGGTGGGGTCTTTCA	
	Tg-mOCT4	TCTCCCATGCATTCAAACTG/ CCTACAGGTGGGGTCTTTCA	
Pluripotency Markers (qPCR)	hDNMT3B	GCTCACAGGGCCCGATACTT/ GCAGTCCTGCAGCTCGAGTTTA	
	hDPPA4	TGGTGTCAGGTGGTGTGTGG /CCAGGCTTGACCAGCATGAA	
	hREX1	CCTGCAGGCGGAAATAGAAC/ GCACACATAGCCATCACATAAGG	
	hSALL2	TCGCTGAGAAGCCCATCATAGA/ TTCCTAGGGTTGGGTCACCAAT	
	hNANOG	ACAACTGGCCGAAGAATAGCA/ GGTTCCCAGTCGGGTTCAC	
	endo-hOCT4	GGGTTTTTGGGATTAAGTTCTTCA/ GCCCCCACCCTTTGTGTT	
	endo-hSOX2	CAAAAATGGCCATGCAGGTT/ AGTTGGGATCGAACAAAAGCTATT	
House-Keeping Gene (qPCR)	GAPDH	GCACCGTCAAGGCTGAGAAC/ AGGGATCTCGCTCCTGGAA	

fenilindol (Martí et al., 2013).

3.5. In vitro and in vivo differentiation

In vitro differentiation was promoted by EB formation. iPSCs colonies were lifted manually and incubated in ultra-low attachment plates in mTeSR1 medium. After 24 h, medium was changed to differentiation medium for additional 24–48 h (Ectoderm: 50% Neurobasal medium, 50% DMEM/F12, 1% N2, 1% B27, 1% Glutamax and 1% P-S; Endoderm: Knockout-DMEM, 10% FBS-Hyclone, 1% NEAA, 0.1% β-mercaptoethanol, 1% Glutamax and 1% P-S; Mesoderm: Endoderm medium supplemented with 0.5 mM ascorbic acid). EBs were seeded on matrigel-coated slide flasks and cultured in differentiation media for 15–20 days, then analyzed by immunocytochemistry as described. Confocal images were taken using Leica TSC SPE or Leica SP5 microscopes.

In vivo differentiation was induced by intratesticular injection of $0.5x10^6$ iPSCs into severe combined immunodeficient (SCID) mice. After 8 weeks of growth, teratomas were extracted, PFA-fixed, paraffin embedded and analyzed by immunocytochemistry.

3.6. Confirmation of microduplications and STR analysis

7q11.23 microduplications and the absence of novel rearrangements were confirmed by SNP-array (Illumina Infinium HumanCore-24, IMPPC) using 0.5 μg of iPSC genomic DNA. Line identities were corroborated by genotyping (STRs-study).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2020.102092.

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