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Lab resource: Stem cell line

Generation of integration free induced pluripotent stem cells from fibrodysplasia ossificans progressiva (FOP) patients from urine samples

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ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is an extremely rare, autosomal dominant transmitted genetic disease. Patients experience progressive bone formation replacing tendons, ligaments, muscle and soft tissue. Cause of FOP are gain-of-function mutations in the Bone Morphogenetic Protein (BMP) receptor *Activin A receptor type 1* (*ACVR1*) (Kaplan et al., 2008). The most common mutation is R206H, which leads to the substitution of codon 206 from arginine to histidine (Shore et al., 2006).
Here, we describe the derivation and characterization of two hiPSC lines from two FOP patients, both carrying the mutation R206H. Cells were isolated from urine and reprogrammed using integration free Sendai virus vectors under defined conditions.

1. Resource table

Name of Stem Cell construct	BCRTi001-A, BCRTi002-A
Institution	Charité—Universitätsmedizin Berlin, Berlin Brandenburg Center for Regenerative Therapies (BCRT)
Person who created resource	Laura Hildebrand
Contact person and email	Harald Stachelscheid (Harald.Stachelscheid@charite.de)
Date archived/stock date	March and June, 2015
Origin	Cells isolated from urine
Type of resource	induced pluripotent stem cells (iPSC) derived from FOP patients
Sub-type	hiPSC cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4
Authentication	STR Analysis/Fingerprinting (available in hPSCreg under controlled access) Sequencing of the mutation
Link to related literature (direct URL links and full references)	
Information in public databases	http://hpscreg.eu/cell-line/BCRTi001-A http://hpscreg.eu/cell-line/BCRTi002-A

Resource details

2. Materials and methods

2.1. Ethics statement

This work was approved by the Ethics Commission of the Charité—Universitätsmedizin Berlin (EA2/047/14).

2.2. Isolation of cells from urine

Isolation of cells from urine was performed as described by Zhou et al. (2012). Briefly, to obtain urinary cells, approximately 100 to 200 ml of urine were collected and centrifuged at 400 *g* for 10 min. The supernatant was discarded and the cell pellet was washed with 10 ml PBS containing 100 U/ml of penicillin and 100 μg/ml streptomycin (Lonza). Following another centrifugation, the pellet was resuspended in 2 ml of primary medium (Dulbecco's Modified Eagle Medium (DMEM) high glucose (Life Technologies) and Ham's F12 (Biochrom) (1:1), 10% (vol/vol) Fetal Bovine Serum (FBS) (Biochrom), 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza), 100 μg/ml normocin (InvivoGen) and the Renal Cell Growth Medium (REGM) SingleQuot kit supplements (Lonza)) and seeded in one well of a 12-well plate (Corning) coated with 0,1% (w/t) gelatin (Gibco). The following three

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days 1 ml of primary medium was added. Four days after plating most of the medium was removed and replaced by renal epithelial/mesenchymal cell (RE/MC) proliferation medium (1:1 mixture of Renal Cell Growth Medium (REBM) medium supplemented with REGM SingleQuots (Lonza) and DMEM high glucose supplemented with 10% (vol/vol) FBS, 1% (vol/vol) GlutaMAX, 1% (vol/vol) non-essential amino acids (NEAA) (all Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml platelet derived growth factor AB (PDGF-AB) and 5 ng/ml human epidermal growth factor (hEGF) (all Peprotech)). Subsequently, half of the culture medium was changed every day. Once cells reached 90% density, they were passaged using TrypLE Select (Life Technologies) with a splitting ratio of 1:4–1:6 and expanded for a maximum of five passages.

2.3. Reprogramming

Reprogramming was performed using Sendai virus vectors (CytoTune-iPS 2.0 Reprogramming Kit, Life Technologies) (Fusaki et al., 2009). Briefly, 5×10^4 cells, seeded in one well of a 24-well plate coated with gelatin and cultured in RE/MC proliferation medium, were transduced using the vectors polycistronic Klf4–Oct3/4–Sox2, cMyc, and Klf4. After addition of the virus, the plate was centrifuged at 800 *g* for 20 min at room temperature. After 24 h medium was replaced by fresh RE/MC proliferation medium and cells were cultured for seven days with medium changes every other day. On day 8 after transduction, cells were passaged using TrypLE Select and seeded with a density of 3×10^4 cells/well in RE/MC proliferation medium onto a 6-well plate coated with Geltrex (Life Technologies). On the next day medium was changed to Essential 8 (E8) medium (Life Technologies) supplemented with100 U/ml penicillin, 100 μg/ml streptomycin and replaced every day until hiPSC colonies appeared. Individual colonies were picked using a pipette tip and expanded in E8 medium on plates coated with Geltrex.

2.4. Test for absence of the reprogramming vector

After passage 12, iPSC lines were tested for absence of the Sendai reprogramming vectors by performing a RT-PCR, which detects the Sendai virus (SeV) genome and the transgenes. For that, total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen) following manufacturer's instructions and cDNA was generated by reverse transcription using the TaqMan Reverse Transcription Reagents (Applied Biosystems). RT-PCR were performed as suggested by the manufacturer of the reprogramming kit (Life Technologies):

95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, finally 72 °C for 10 min. The primer sequences for Sendai virus genome (SeV), krueppel-like factor 4—transgene (SeV-Klf4), cMyc—transgene (SeV-cMyc), and Klf4-Oct 3/4-SOX2—transgene (SeV-KOS) are given in the table below (Table 1). As an internal control the housekeeping gene for the 18 s ribosomal RNA was detected. PCR products were analyzed by 2% agarose gel electrophoresis (Life Technologies) (Fig. 2).

Table 1
List of primers used for Sendai testing, germ layer PCR.

Target		Primer sequence	Product size [bp]
Sendai testing			
SeV	Forward	GGA TCA CTA GGT GAT ATC GAG C	181
	Reverse	ACC AGA CAA GAG TTT AAG AGA TAT GTA TC	
SeV-Klf4	Forward	TTC CTG CAT GCC AGA GGA GCC C	410
	Reverse	AAT GTA TCG AAG GTG CTC AA	
SeV-cMyc	Forward	TAA CTG ACT AGC AGG CTT GTC G	532
	Reverse	TCC ACA TAC AGT CCT GGA TGA TGA TG	
SeV-KOS	Forward	ATG CAC CGC TAC GAG TGA GCG C	528
	Reverse	ACC TTG ACA ATC CTG ATG TGG	
Hu18SRNA	Forward	GTA ACC CGT TGA ACC CCA TT	151
	Reverse	CCA TCC AAT CGG TAG TAG CG	
Pluripotency and differentiation marker			
Nanog	Forward	AAGGTCCCGGTCAAGAAACAG	237
	Reverse	CTTCTGCGTCACACCATTGC	
Brachyury	Forward	TAAGGTGGATCTTCAGGTAGC	252
	Reverse	CATCTCATTGGTGAGCTCCCT	
Goosecoid	Forward	AACGCGGAGAAGTGGAACAAG	89
	Reverse	CTGTCCGAGTCCAAATCGC	
Mixl1	Forward	CTGTTCCCCTCTCTCTGAAGA	67
	Reverse	GGCAGAAAAGATGTGTTCCTCC	
Foxa2	Forward	GGAGCAGCTACTATGCAGAGC	83
	Reverse	CGTGTTCATGCCGTTCATCC	
Sox17	Forward	GTGGACCGCACGGAATTTG	94
	Reverse	GGAGATTCACACCGGAGTCA	
Nestin	Forward	TTGCCTGCTACCCTTGAGAC	145
	Reverse	GGGCTCTGATCTCTGCATCTAC	
Sox1	Forward	CAGTACAGCCCCATCTCCAAC	287
	Reverse	GCGGGCAAGTACATGCTGA	
NeuroD	Forward	GCCCCAGGGTTATGAGACTATCACT	523
	Reverse	CCGACAGAGCCCAGATGTAGTTCTT	
Otx2	Forward	TGTAGAAGCTATTTTTGTGGGTGA	98
	Reverse	GAGCATCGTTCCATCTAACTTTTT	
Pax6	Forward	TGTCCAACGGATGTGTGAGT	162
	Reverse	TTTCCCAAGCAAAGATGGAC	

2.5. Culture of iPSC

The hiPSC were cultured in E8 medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in 6 well dishes coated with Geltrex and routinely passaged using 0.5 mM EDTA (Life Technologies) in PBS every 5–7 days at a ratio of ~1:10.

2.6. Immunofluorescence staining for pluripotency markers

For staining hiPSC were grown on 96 well imaging plates (CellCarrier, Perkin Elmer) in E8 medium. After three days, cells were fixed with Cytofix reagent, followed by blocking and permeabilization with PermWash reagent (both BD) supplemented with 5% FBS. Then, cells were incubated with dye-conjugated antibodies for octamer transcription factor 3/4 (Oct3/4) (PerCP-Cy7, BD, 1:100), stage specific embryonic antigen 4 (SSEA4) (FITC, R&D; 1:100), tumor rejection antigen 1–60 (Tra-1-60) (DyLight 550, Novus Biologicals, 1:100) and Tra-1-81 (DyLight 650, Novus Biologicals, 1:100). Nuclei were stained with Hoechst 33,342 (2.5 μg/ml in PBS, Invitrogen). Microscopic analysis was performed with an Operetta high content imaging system (Perkin Elmer) (Fig. 1).

2.7. In vitro differentiation into the three germ layers

Undirected differentiation of iPSC into the three germ layers was performed by generation of embryoid bodies (EBs) using AggreWell plates (StemCell Technologies). EBs were cultured in DMEM Low Glucose supplemented with 10% FBS, 2 mM/ml L-Glutamine, 100 U/ml of penicillin and 100 μg/ml streptomycin for 10 days. Medium was changed every other day.

2.8. RT-PCR of markers for pluripotency and the three germ layers

500 ng of total RNA from EBs was reverse transcribed into cDNA. 1 μl of this reaction was subjected to Polymerase Chain Reaction (PCR) using primers specific for NANOG, brachyury, goosecoid, mix paired-like homeobox (Mixl1), forkhead-box-protein A2 (FoxA2), 6 = SRY (Sex Determining Region Y)-Box 17 (Sox17), Sox1, nestin, NEUROD, orthodenticle homeobox 2 (Otx2), and Paired box protein 6 (Pax6)

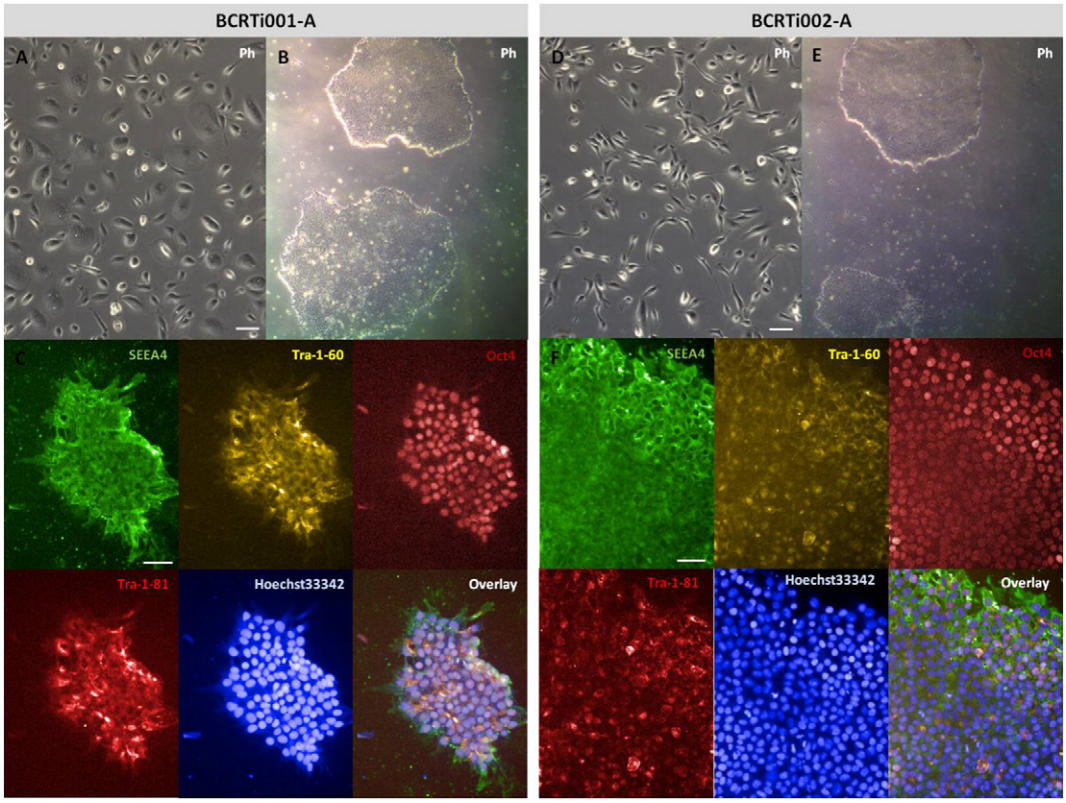


Fig. 1. Morphology of primary cells, iPSC and expression of pluripotency markers in iPSC. [A + D] Phase contrast images of primary cells derived from urine. [B + E] Phase contrast images of reprogrammed iPSC. [C + F] Staining of pluripotency markers Anti-stage specific embryonic antigen 4 (SSEA4) (cell surface), Anti-Tra-1-60 (cell surface), Anti-Oct3/4 (nuclear), Anti-Tra-1-81 (cell surface), Hoechst33342 (nuclear) and overlay of all markers. (Upper scale bar = 100 μm, lower scale bar = 50 μm).

(see Table 1). Cycle program was the following: 96 °C for 5 min, 35 cycles of 96 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Final step was 72 °C for 7 min. PCR products were analyzed using a by electrophoresis in an 2% agarose gel (Fig. 3).

2.9. Sequencing

To confirm presence of the FOP mutation R206H in the hiPSC, sequencing was performed. For that, genomic DNA (gDNA) was isolated

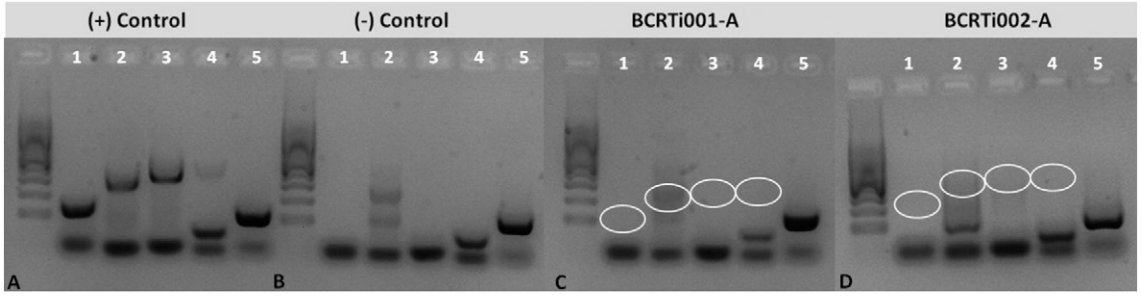


Fig. 2. Test for absence of Sendai virus. RT-PCR for reprogramming vectors in [A] positive control [B] negative control [C] BCRTi001-A and [D] BCRTi002-A. 1 = Sendai virus (SeV), 2 = krueppel-like factor 4—transgene (SeV-Klf4), 3 = cMyc—transgene (SeV-cMyc), 4 = Klf4-Oct 3/4-SOX2—transgene KOS (SeV-KOS), 5 = 18 sRNA control.

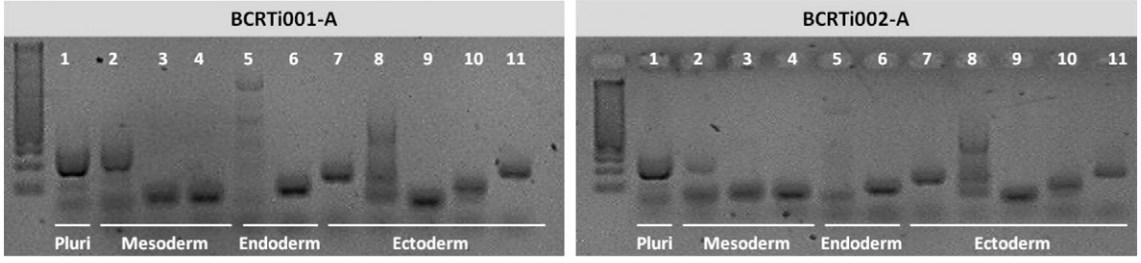


Fig. 3. Differentiation capacity of iPSC into all three germ layers. RT-PCR for pluripotency and germ layer markers for [A] BCRTi001-A and [B] BCRTi002-A. 1 = Nanog, 2 = Brachyury, 3 = Goosecoid, 4 = Mix Paired-Like Homeobox (Mixl1), 5 = Forkhead-Box-Protein A2 (FoxA2), 6 = SRY (Sex Determining Region Y)-Box 17 (Sox17), 7 = Nestin, 8 = Sox1, 9 = NeuroD, 10 = Orthodenticle homeobox 2 (Otx2), 11 = Paired box protein 6 (Pax6).

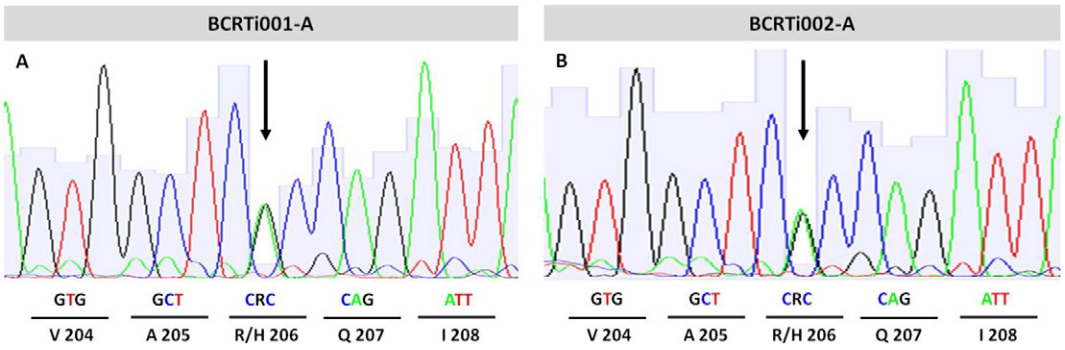
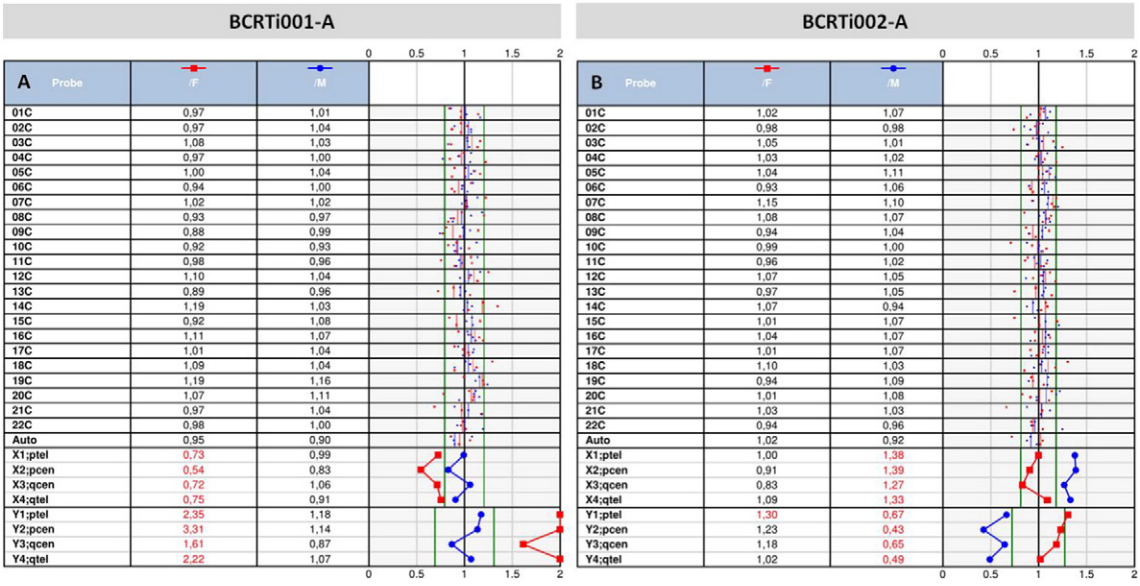


Fig. 4. Sequencing of FOP mutation R206H. Heterozygous mutation Guanine to Adenine, leading to exchange of amino acid Arginine to Histidine at position 206 in amino acid sequence was confirmed in [A] BCRTi001-A and [B] BCRTi002-A.



A Probe	/F	/M
01C	0,97	1,01
02C	0,97	1,04
03C	1,08	1,03
04C	0,97	1,00
05C	1,00	1,04
06C	0,94	1,00
07C	1,02	1,02
08C	0,93	0,97
09C	0,88	0,99
10C	0,92	0,93
11C	0,98	0,96
12C	1,10	1,04
13C	0,89	0,96
14C	1,19	1,03
15C	0,92	1,08
16C	1,11	1,07
17C	1,01	1,04
18C	1,09	1,04
19C	1,19	1,16
20C	1,07	1,11
21C	0,97	1,04
22C	0,98	1,00
Auto	0,95	0,90
X1;ptel	0,73	0,99
X2;pcen	0,54	0,83
X3;qcen	0,72	1,06
X4;qtel	0,75	0,91
Y1;ptel	2,35	1,18
Y2;pcen	3,31	1,14
Y3;qcen	1,61	0,87
Y4;qtel	2,22	1,07

B Probe	/F	/M
01C	1,02	1,07
02C	0,98	0,98
03C	1,05	1,01
04C	1,03	1,02
05C	1,04	1,11
06C	0,93	1,06
07C	1,15	1,10
08C	1,08	1,07
09C	0,94	1,04
10C	0,99	1,00
11C	0,96	1,02
12C	1,07	1,05
13C	0,97	1,05
14C	1,07	0,94
15C	1,01	1,07
16C	1,04	1,07
17C	1,01	1,07
18C	1,10	1,03
19C	0,94	1,09
20C	1,01	1,08
21C	1,03	1,03
22C	0,94	0,96
Auto	1,02	0,92
X1;ptel	1,00	1,38
X2;pcen	0,91	1,39
X3;qcen	0,83	1,27
X4;qtel	1,09	1,33
Y1;ptel	1,30	0,67
Y2;pcen	1,23	0,43
Y3;qcen	1,18	0,65
Y4;qtel	1,02	0,49

Fig. 5. Karyotyping of the cell lines. Karyotyping was performed using the KaryoLite™ BoBs™ assay. Both iPSC lines (a) BCRTi001-A, (b) BCRTi002-A showed a normal karyotype. The red and blue lines indicate chromosomal signal ratios against female (red) and male (blue) reference DNA with normal karyotypes. For the normal chromosomes both the ratios against female and male references should be in the reference area (green lines) around a value of 1, whereas for the abnormal karyotype both signals should be outside the reference area.

using the Genomic DNA tissue kit (Machery-Nagel) following the instructions. 50 ng of gDNA served as a template in a touch-down PCR with this program: 94 °C for 30 s, followed by 25 cycles of 95 °C for 30 s, 59–56 °C for 30 s and 68 °C for 30 s, finally 68 °C for 7 min (primer sequences F: CCAGTCCTTCTTCCTTCTTCC, R: AGCAGATTTTCCAAGTTCCATC) (Shore et al., 2006). Quality of amplification products was analyzed using a 1% agarose gel and sequenced using a 3730 DNA Analyzer (Life Technologies). Resulting sequence information was analyzed with DNASTAR software (Fig. 4).

3. Verification and authentication

3.1. Karyotyping

Karyotyping was carried out using KaryoLite™ BoBs™ (Perkin Elmer) (Lund et al., 2012). From each sample at least 240 ng of gDNA was used as a starting material. The samples were processed according to the manufacturer's instructions. Briefly, gDNA was labelled with Biotin, purified and hybridized to beads with complementary bacterial artificial chromosome (BAC) probes. After washing and reporter (streptavidin-PE) binding, the fluorescent signals were measured with a Bio-Plex 200 system (BioRad). Results were analyzed using BoBsoft™ analysis software (Perkin Elmer). As references female and male gDNA (Promega) were used. A sample was defined as 'normal disomic', when the fluorescent ratio was ~1.0 for all loci analyzed (Fig. 5).

3.2. Fingerprinting

For identity and purity verification of the iPSC lines, Short Tandem Repeat (STR) analysis was performed from the primary cells and the reprogrammed iPSC by amplification of 10 loci from the Combined DNA Index Systems (CODIS), namely D16S539, D7S820, CSF1PO, D5S818, TPOX, D13S317, vWA, HUMTH01, DXS101 and DYS393. For personalized data protection reasons, resulting data are only available in the hPSCreg database under controlled access.

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